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INVESTIGATING THE ROLE OF P73 PROTEINS IN CONTROLLING THE TUMOR MICROENVIRONMENT

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Stockholm 2020

Published by Karolinska Institutet.
Printed by Eprint AB 2020

ISBN 978-91-7831-929-9

Investigating the Role of p73 Proteins in Controlling the Tumor Microenvironment

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Publicly defended at Karolinska Institutet,
Lecture Hall Gustav Retzius, Berzelius väg 3,
Solna Campus

Friday, November 13th, 2020, 09.30

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To my family and friends, who have supported me throughout these years.

Nothing in life is to be feared,
it is only to be understood.

- Marie Curie

ABSTRACT

Cancer is a multifactorial disease governed by oncogenes and tumor suppressors that not only impact on the behavior of the cancer cells but also influence many processes in the surrounding tumor microenvironment. The most studied tumor suppressor family is the p53 family, which consists of the transcription factors p53, p63 and p73. While p53 is the most frequently mutated gene in the cancer genome, for both p63 and p73, a shift in the balance between different isoforms has been discovered and this deregulation of protein levels has been linked to tumor progression and survival. P73 can be transcribed from two separate promoters resulting in a tumor suppressing, full-length isoform (TAp73), and a N-terminally truncated version (ΔNp73), which lacks the transactivation domain and thus possesses oncogenic properties. Furthermore, alternative splicing in the C-terminus results in a number of additional isoforms. P73 has been shown to be able to support and overtake many processes p53 is regulating. However, p73 isoforms have also been shown to have p53-independent functions. Understanding how different p53 family members regulate tumor development and progression is essential for identifying possible treatment strategies.

In this thesis we identified several previously unknown roles for p73 isoforms in controlling the tumor microenvironment. Firstly, we discovered that loss of TAp73 results in a NF-κB-dependent upregulation of pro-inflammatory factors in breast cancer. Furthermore, this led to a concurrent increase in tumor-promoting macrophage infiltration. Secondly, we identified a role for ΔNp73 in the regulation of activating NK cell ligand expression on cancer cells. However, we observed a concomitant upregulation of inhibitory NK cell ligands upon loss of ΔNp73, leaving NK cell-mediated killing of tumor cells unaffected. Thirdly, a correlation between high levels of ΔNp73 and HIF-1α protein was observed. We demonstrate that ΔNp73 increases HIF-1α protein stability by interfering with the expression of genes of the ECV complex, normally involved in proteasomal degradation of HIF-1α. Finally, we further strengthen the involvement of ΔNp73 in the process of multidrug resistance. ΔNp73 was found to promote elevated expression of ABC transporters, ABCB1 and ABCB5, in breast cancer and melanoma, thereby supporting the efflux of drugs from the cancer cells and increasing their resistance to drug treatments.

Taken together, these findings highlight the significant contributions of p73 isoforms on tumor progression and aid in unravelling the complex interactions of this network.

LIST OF SCIENTIFIC PAPERS

I. **Johanna Wolfsberger**, Habib A. M. Sakil, Leilei Zhou, Veronica Zubillaga, Sabrina de Souza Ferreira, Marina Stantic, Nicolas Fritz, Johan Hartman, Charlotte Rolny and Margareta T. Wilhelm.
TAp73 represses NF-κB-mediated recruitment of tumor-associated macrophages in breast cancer.
Submitted Manuscript, under revision

II. **Johanna Wolfsberger**, Dhifaf Sarhan, Niek Van Bree, and Margareta T. Wilhelm.
Loss of ΔNp73 leads to an increase in NKG2D ligand expression, while not affecting NK cell-mediated tumor cell killing in breast cancer.
Manuscript

III. Marina Stantic, **Johanna Wolfsberger**, Habib AM Sakil, Margareta T. Wilhelm,
ΔNp73 enhances HIF-1α protein stability through repression of the ECV complex.
Oncogene, 2018 Jul;37(27):3729-3739.

IV. Habib AM Sakil, Marina Stantic, **Johanna Wolfsberger**, Suzanne E. Brage, Johan Hansson, Margareta T. Wilhelm.
ΔNp73 regulates the expression of the multidrug-resistance genes ABCB1 and ABCB5 in breast cancer and melanoma cells - a short report.
Cell Oncol (Dordr). 2017 Dec;40(6):631-638.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ADCC	Antibody-mediated cellular cytotoxicity
Arg1	Arginase 1
CAF	Cancer associated fibroblast
CBP	CREB-binding protein
CCL2	C-C motif chemokine ligand 2
CCL5	Chemokine (C-C motif) ligand 5
CCR2	C-C chemokine receptor type 2
CSF-1	Colony stimulating factor 1
CSF1R	Colony stimulating factor 1 receptor
DAMP	Danger associated molecular pattern
DBD	DNA binding domain
DCIS	Ductal carcinoma in situ
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
g-gp	g-glycoprotein
GM-CSF	Granulocyte-macrophage colony stimulating factor
HER2	Human endothelial growth factor
HIF-1α	Hypoxia inducible factor 1 α
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HR	Hormone receptor
HRE	Hypoxia response elements
IFN-γ	Interferon γ
IKKβ	IkappaB kinase β
IL-1	Interleukin 1

IL-10	Interleukin 10
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-2	Interleukin 2
IL-4	Interleukin 4
IL4R	IL-4 receptor
IL-6	Interleukin 6
KIR	Killer-cell immunoglobulin-like receptor
LCIS	Lobular carcinoma in situ
LPS	Lipopolysaccharide
MARCO	Macrophage receptor with collagenous structure
MDM2	Mouse double minute 2 homolog
MDR1	Multidrug resistance gene 1
MDSC	Myeloid derived suppressor cell
MEFs	Mouse embryonic fibroblasts
MHC	Major histocompatibility complex
MICA	MHC class I chain-related protein A
MICB	MHC class I chain-related protein B
MMP9	Matrix metalloprotease 9
NCR	Natural cytotoxic receptor
NK cell	Natural killer cell
OD	Oligomerization domain
PAMP	Pathogen associated molecular pattern
PD-1	Programmed cell death protein 1
PDGFB	Platelet-derived growth factor B
PD-L1	Programmed cell death ligand 1
PGE2	Prostaglandin E2
PHD	Prolyl hydroxylase

PR	Proline rich sequences
PRR	Pattern recognition receptor
Rb	Retinoblastoma
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAM	Sterile motive alpha domain
SIRPα	Signal regulatory protein α
SV40	Simian Vacuolating Virus 40
TAD	Transactivation domain
TAM	Tumor associated macrophage
TCGA	The cancer genome atlas
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor α
Treg	Regulatory T cell
ULBP	UL binding protein
VEGFA	Vascular endothelial growth factor A
VEGFC	Vascular endothelial growth factor C
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel-Lindau protein
WHO	World Health Organization
YAP-1	Yes-associated protein

1 INTRODUCTION

1.1 CANCER

According to the World Health Organization (WHO) cancer is the second leading cause of death worldwide with every sixed death caused by cancer [1]. A tumor is defined as a mass of transformed cells proliferating at an abnormal rate, which can be benign or malignant. Malignant tumors are considered cancerous and can invade the surrounding tissues as well as spread to other organs resulting in metastasis.

Multiple factors play a role in the development and progression of cancer. Ten hallmarks have been proposed to be essential for this process, including perpetual growth factor signaling, avoiding cell death through apoptosis, changes in cellular metabolism and the continuous accumulation of mutations through genetic instability (see Figure 1) [2].

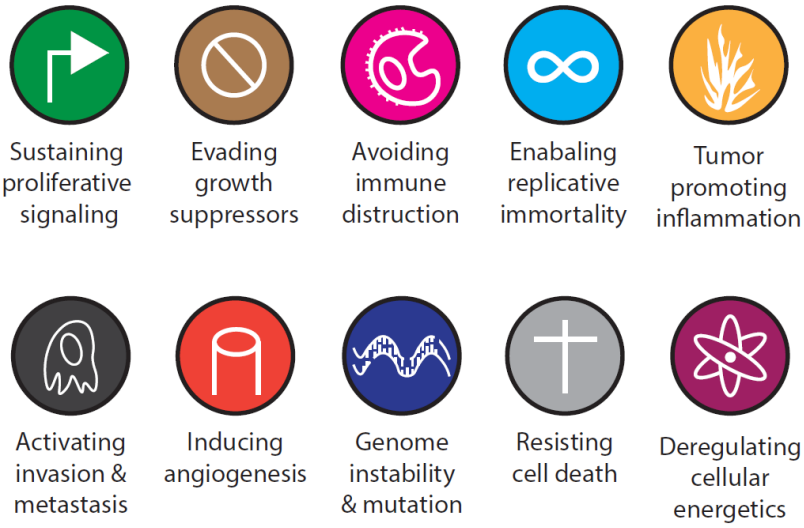

Figure 1. The hallmarks of cancer by Hanahan and Weinberg.

Undeniably, one crucial factor tightly linked to cancer development and progression is the body's own immune system [3]. Other factors such as oxygen regulation and angiogenesis are also attributed a vast impact on cancer establishment [4]. These fields of research together with multidrug resistance will be discussed throughout this thesis.

Despite great achievements in the field of cancer research during recent years, numerous elements remain unknown and further detailed understanding is needed to reach a point where there can be effective treatment, or ultimately even a cure, for all people suffering from cancer.

1.1.1 Oncogenes and Tumor Suppressors

Under normal circumstances, cell growth and division are tightly regulated processes where multiple checkpoints must be passed before progression to prevent replication of faulty DNA. Mutations in key regulator genes can lead to deviation in these processes and might result in unlimited and unregulated cell division, regardless of errors in DNA replication.

Oncogenes

Oncogenes were discovered by chance while studying RNA viruses. It was found that some viruses were able to transform normal cells to become cancerous. With time it was understood that these viruses carry genes that interfere with cellular processes and induce uncontrolled proliferation. These genes were called viral oncogenes. Back then, it was believed that all tumors derive from viral infections. However, later it was shown that homologues of most of these viral genes were present in the cellular genome and that viruses were not essential for tumor development [5]. This meant that we carry multiple so-called proto-oncogenes in our genome, that can be converted into oncogenes and induce tumorigenesis. Often proto-oncogenes belong to classes of genes that regulate cell growth and differentiation and their activation is strictly limited to specific events during cellular development. Proto-oncogenes can be growth factors (EGF, VEGF), growth factor receptors (EGFR, VEGFR), signal transducers (RAS, BRAF), protein kinases (SRC, ABL, AKT) and nuclear oncogenes/transcription factors (MYC, HIF) [6,7]. Upon changes in genetic configuration (such as point mutations, amplifications or chromosomal rearrangements) these genes can become constantly activated and turn into oncogenes as which they promote uncontrolled growth [8]. Oncogenes are therefore commonly referred to as the gas paddles of the cells.

Tumor suppressors

Conversely, tumor suppressors are usually called the brakes of the cells. Upon loss of their function cells gain the ability to overcome the strict regulation of cellular growth and division and gain the ability to replicate without hindrance. Tumor suppressors are genes that are often involved in processes such as DNA repair and cell cycle control. Unlike oncogenes, both alleles of the tumor suppressor gene must be altered to result in significant consequences for the cell. This phenomenon is referred to as Knudson's two-hit hypothesis. Tumor suppressor genes can be dysfunctional due to deletion or inactivation, but also by epigenetic silencing or proteasomal degradation [9]. However, there are exceptions to this rule. One example will be discussed later in the section P73.

Undisputable, the most famous tumor suppressor up to date is p53, which will be discussed in more detail in the section 'P73 – Part of the p53 family' [10]. Other major tumor suppressor genes are retinoblastoma (Rb), p16, PTEN, BRCA1/2, CDKN2A and VHL [7,11,12], which are involved in regulating cell cycle progression, DNA damage sensing or are negative regulators of proto-oncogenes.

1.1.2 Breast Cancer

Breast cancer is the second most common cancer worldwide and the most common cancer in women (see Figure 2). Even though tremendous efforts have been made to fight this disease still in 2018 an estimate of 627,000 women died from breast cancer [13].

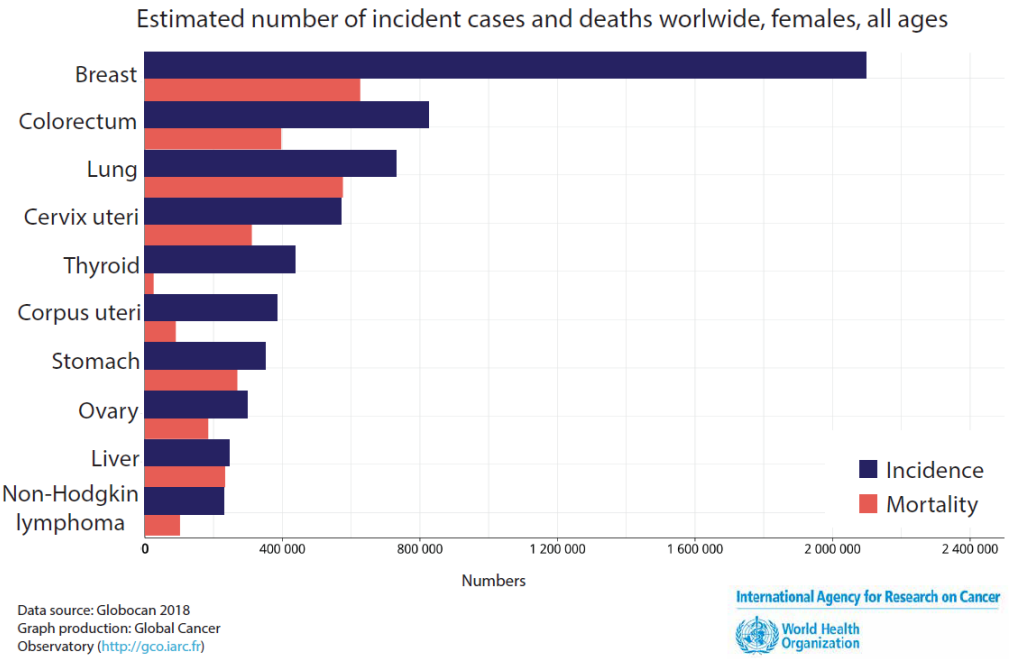

Figure 2. Cancer incidences in females worldwide.

Overall, even though a rise in incidence rates can be observed, breast cancer deaths have declined dramatically over the last three decades by up to 40 percent. This is mainly due to the implementation of screening programs that allow for detection of cancer at an earlier stage, but also due to advances in cancer therapies [14]. Compared to other cancers, breast cancer now shows a rather good prognosis. In high-income countries the overall five-year survival rate for women diagnosed with breast cancer lies around 91 percent, while ten-year survival reaches 84 percent [15]. However, the statistics vary dramatically in low- and middle-income countries where breast cancer is often detected at a later stage.

Anatomically, breast cancer can arise in the ducts (ductal carcinoma in situ (DCIS)) or in the lobes (lobular carcinoma in situ (LCIS)) of the breast tissue (see Figure 3). However, only DCIS has been found to progress to the stage of invasive breast cancer [16].

First line of treatment for breast cancer consists of surgery, either mastectomy (complete removal of the breast) or breast-conserving surgery (removing only the tumor and surrounding tissue). Surgery is often accompanied by radiation, chemotherapy, hormonal or targeted therapy. Recently, for the first time an immunotherapeutic drug, namely a monoclonal antibody against the immune checkpoint protein PD-L1, in combination with chemotherapy has been

approved for triple negative breast cancer [17]. Which treatment is selected depends on many factors, including the type, stage and spread of the breast cancer and the patient's age.

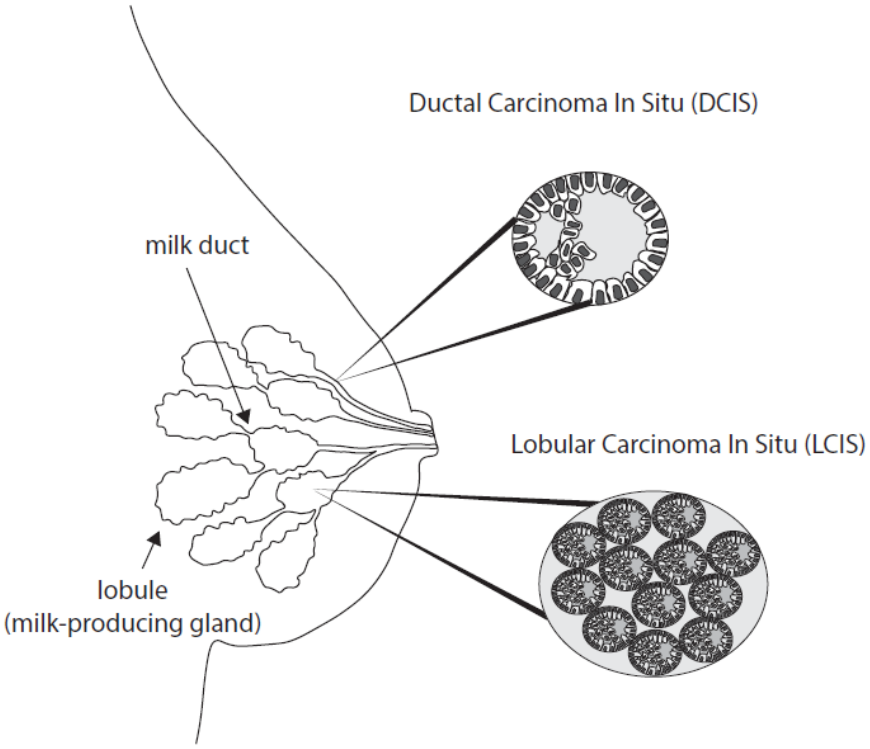

Figure 3. Anatomic location of breast cancer

Subtypes of breast cancer

Broadly, breast cancer can be classified into four molecular subtypes that differ greatly in prognosis and treatment options [18].

Luminal A (HR+/HER2-)

The majority of breast cancers, almost 75 percent, belong to the group of hormone receptor (HR) positive breast cancers. These cancers are defined by the expression of estrogen and/or progesterone receptors (ER/PR) on the cell surface [19]. ER is a nuclear hormone receptor that functions as a transcription factor [20]. PR is downstream of the ER signaling pathway, thus their expression usually correlates [21]. Typically, the Luminal A subtype has a rather good prognosis due to a general good response to hormonal therapies. Patients with ER-positive cancers usually benefit from endocrine therapy which is targeting ER, such as Tamoxifen and aromatase inhibitors [22]. However, these breast cancers usually do not respond well to chemotherapies [23].

Luminal B (HR+/HER2- or HR+/HER2+)

Breast cancers of the Luminal B type are classified by the expression of HRs, however show lower levels than the Luminal A type. Additionally, some Luminal B tumors express the human epidermal growth factor 2 receptor (HER2). HER2 is a tyrosine kinase receptor located in the cell membrane, which is involved in cell proliferation and survival. In breast cancer, HER2 is often upregulated due to gene amplification or overexpression. Autophosphorylation of HER2 heterodimers leads to the activation of signaling pathways important for cell proliferation and survival, including PI3K/AKT and RAS/RAF/MEK/ERK [24]. Usually, the Luminal B subtype is also highly positive for Ki67 (>20%), a marker for rapid cell proliferation, which correlates with poor outcome [25]. This leaves Luminal B cancers with a worse prognosis compared to the Luminal A subtype [26].

HER2-enriched (HR-/HER2+)

A small percentage of breast cancers show expression of HER2 without concomitant expression of HRs. Even though this group had the worst prognosis before, the current standard treatment are targeted therapies against HER2, which led to a drastic improvement of survival. So far, a few distinct options have been approved to target HER2: humanized monoclonal antibodies, such as trastuzumab; small molecular receptor tyrosine kinase inhibitors; and an antibody drug conjugate of trastuzumab [27-29].

Triple negative breast cancer (HR-/HER2-)

The final group is a heterogeneous group termed triple negative breast cancer (TNBC), which is defined by the absence of HR and HER2 expression and accounts for around 15-20% of all breast cancers [30]. This group can be further divided into prognostically significant subtypes depending on their gene expression, including basal-like 1, basal-like 2 and immunomodulatory. Actually, 80% of TNBCs show mutations in p53 and p53 expression has been correlated with poor prognosis [31].TNBCs are considered to have the worst prognosis of all breast cancer subtypes, showing a five-year survival rate of around 77 percent. Unfortunately, so far, systemic treatment options are limited to different cytotoxic agents, highlighting a great need for improved therapeutic interventions [32].

1.1.3 Malignant Melanoma

Uncontrolled proliferation of melanocytes that are located in the basal layer of the epidermis can give rise to melanoma. The incidence of melanoma has increased drastically over the last half century, with a clear upwards trend in Australia, Europe and North America. Representing only five percent of all skin cancers, melanoma is classified as the most aggressive type of skin cancer with median survival times of 8-12 months and it accounts for most of skin cancer-related deaths [33]. If diagnosed early, simple surgery can resolve the problem. However, due to its aggressive nature melanoma usually spreads quickly to other organs, preferentially to the

lungs, liver and brain [34]. Melanoma is often driven by mutations in signaling pathways controlling cell proliferation, growth, apoptosis and replicative lifespan of the cell. Thus, mutations in genes like BRAF, PTEN, P53 and TERT are commonly found in melanoma cells [35]. Furthermore, families carrying hereditary mutations in the CDKN2A gene have an increased risk of developing melanoma [36]. CDKN2A is involved in the regulation of cell cycle progression through governing the G1 checkpoint and inducing stable p53 expression [37]. As it is the case for breast cancer, Ki67 is used as a prognostic marker in melanoma to identify high cell proliferation, which correlates with the aggressiveness of the cancer [38].

Previously, surgery and chemotherapy were the only treatment strategies for melanoma. Today, several targeted therapies are available for the treatment of melanoma. One of the most promising and frequently used strategy is to target BRAF in patients with BRAF mutations, using inhibitors such as vemurafenib [39]. Additionally, immune check point inhibitors were found to have impressive effects regarding long-term survival, at least in a subset of patients, compared to other treatment options. The anti-CTLA-4 antibody ipilimumab and antibodies targeting the PD-1 receptor on T cells, such as pembrolizumab and nivolumab, are standard treatment of care and often used in combination to treat late stage melanoma [40].

Even though these new types of therapies have led to significant improvements regarding overall survival, mortality rates are continuously rising. This is due to increasing numbers of cases as well as rapid development of resistance to therapies such as BRAF inhibitors [41,42], displaying the need for further research on melanoma.

1.1.4 The Tumor Stroma

Unlike one could believe, the tumor site not only consists of rapidly dividing tumor cells. Instead, the surrounding microenvironment of a tumor usually contains a complex mix of cell types of different origin (see Figure 4). Various cells of the innate and adaptive immune system can be found in the tumor microenvironment (TME), such as macrophages, dendritic cells, myeloid derived suppressor cells (MDSCs), NK cells, different subtypes of T cells and even B cells. Furthermore, endothelial cells and pericytes, that compose blood vessels and are essential for angiogenesis, make up an important part of the tumor stroma. Fibroblasts that get converted into cancer-associated fibroblasts (CAFs) which produce collagen fibers and aid in building up the extracellular matrix (ECM), are regular constituents of the tumor stroma [43].

The majority of these cells are components of the normal tissue stroma with intrinsic anti-tumoral capabilities, but are highjacked by the tumor cells to tolerate and even promote tumor growth. Other cells, such as most of the immune cells, get recruited to the tumor site by cytokines and chemokines released by the tumor and are then ‘educated’ by the suppressive environment to support tumor growth [44]. The tumor stroma plays an essential role in tumor development and progression. The constant bilateral exchange of signals between tumor cells and cells of the tumor stroma allows for continuous growth and even invasion and metastasis of the tumor [45].

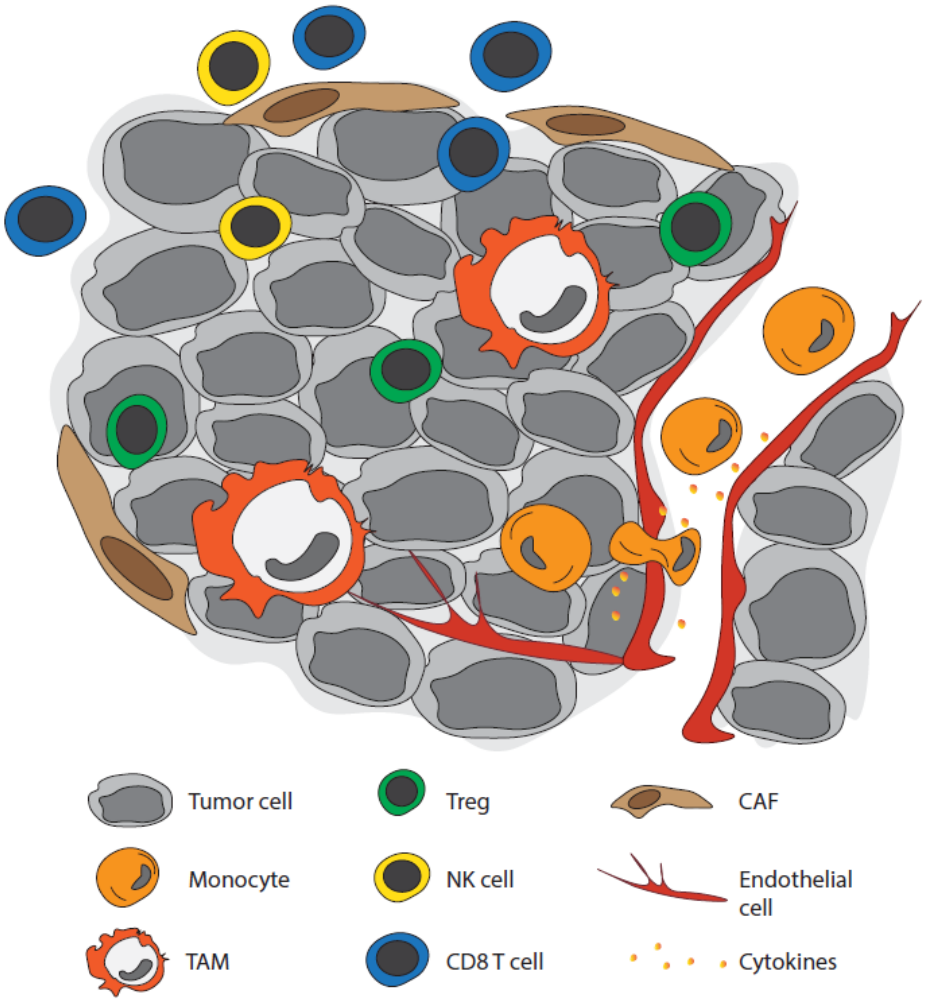

Figure 4. The tumor microenvironment – host to a variety of stromal cells.

Even though each single cell type represented in the tumor stroma is of importance for tumor progression, for the scope of this thesis only macrophages and natural killer cells will be further discussed in detail.

1.1.5 Macrophages

Macrophages belong to the innate branch of the immune system and are involved in a variety of processes assisting in keeping homeostasis in the body. Resident macrophages can be found in almost any tissue and are termed differently depending on their location (Langerhans cells in the skin, Kupffer cells in the liver, microglia in the brain or alveolar macrophages in the lung). On one hand, macrophages can originate from bone marrow-derived monocytes that circulate through the blood and get recruited to the tissue in case of infection or to replenish tissue-resident cells. On the other hand, tissue-resident macrophages can have their origin in the yolk sac and the fetal liver, where all macrophages are produced during embryonic development [46,47].

During infection macrophages participate in the first line of defense. Macrophages possess a range of pattern recognition receptors (PRRs) that can recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) that are intrinsic to pathogens and foreign particles. Upon activation, macrophages release different cytokines and proinflammatory mediators, reactive oxygen/ nitrogen species (ROS/ RNS) and antimicrobial peptides. As their name macrophage (Greek, "big eater") suggests they can engulf pathogens by phagocytosis and lyse them inside phagolysosomes located in their cytoplasm [48]. Macrophages can also act as antigen presenting cells via their MHC class I and II receptors, even though they do so less efficiently than dendritic cells [49].

Macrophages show a high grade of plasticity, implying that they can easily change activation state depending on the factors they receive from their surroundings. Exposed to factors like interferon γ (IFN-γ), lipopolysaccharide (LPS) or granulocyte-macrophage colony stimulating factor (GM-CSF) they acquire a pro-inflammatory (classically activated) state which allows for stimulation of T cells. Surface markers that define this activation state include CD80, CD86 and high expression of MHC class II. On the other hand, if factors like interleukin 4 (IL-4) and IL-13 prevail macrophages turn into an anti-inflammatory (alternatively activated) self which is crucial in tissue maintenance and wound healing [50]. This activation state is accompanied by surface expression of markers such as CD206, CD163, and CD204 [51,52]. However, depending on their surrounding environment macrophages can adapt easily and acquire any stage in between these two extremes [53]. Due to the great plasticity macrophages display there has been an ongoing discussion in the field how to uniformly define different macrophage populations and activation states [54].

1.1.5.1 Tumor-associated macrophages

Macrophages are present in the microenvironment of most cancers and usually constitute a critical mass of the tumor stroma [55]. Importantly, high macrophage infiltration strongly correlates with poor patient survival [56,57]. It has been shown that cancer cells express cytokines and chemokines (e.g. colony stimulating factor 1 (CSF-1) and C-C motif chemokine ligand 2 (CCL2)) that attract bone marrow-derived monocytes from the blood into the tumor microenvironment where they mature into macrophages. These tumor-associated macrophages (TAMs) are exposed to distinct stimuli (IL-4, IL-10, transforming growth factor β (TGF-β)) released by the cancer cells favoring an anti-inflammatory and tumor-promoting state of these cells (see Figure 5) [58]. TAMs, in return, initiate the secretion of pro-tumorigenic cytokines like IL-10, TGF-β and factors such as arginase 1 (Arg1), thereby promoting an immunosuppressive environment and the direct inhibition of cytotoxic T cells. Furthermore, they release factors including vascular endothelial growth factor A (VEGFA) and matrix metalloprotease 9 (MMP9), which promote angiogenesis and result in an increased invasive behavior of the tumor cells [59]. This type of heterotypical signaling between cancer cells and the surrounding stroma is critical for tumor progression [60].

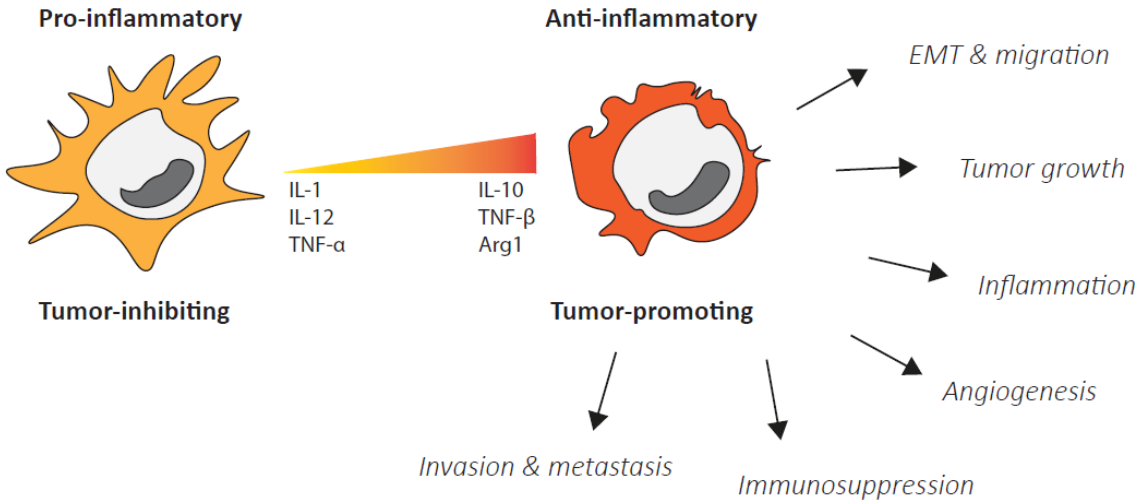

Figure 5. Plasticity of the activation state of macrophages and tumor promotion.

Consistent with their great plastic potential, distinct subsets of TAMs can be encountered in different regions of the TME. Hypoxic areas are found to harbor TAMs with greater tumor-promoting activity which were also defined to be more pro-angiogenic. As the tumor progresses these pro-tumoral TAMs get more abundant and overrule other, less tumor-promoting, TAM populations [61].

1.1.5.2 Targeting macrophages in cancer

Due to the vast impact TAMs have on cancer progression, a lot of focus has been put on targeting macrophages in cancer. Several strategies have been tested, including complete depletion of macrophages, as well as shifting their activation state to a pro-inflammatory profile [62]. Listed below are some of the strategies currently tested in the clinics.

<u>Eliminating TAMs and blocking TAM recruitment</u>

Clodronate and Zoledronic acid are two small molecules belonging to the group of bisphosphonates. They have been shown to effect tumor growth directly and indirectly, by exerting apoptotic effects on macrophages, and are therefore currently investigated in clinical trials [63]. Trabectedin is another small molecule that, besides its direct effects on tumor cells, was found to affect macrophage viability by inducing caspase 8 activation, thereby promoting apoptosis [64].

Additionally, several studies have shown the effectiveness of targeting the CCL2/CCR2 or CSF/CSFR axis to inhibit TAM recruitment [65,66]. However, at least when inhibiting CCL2, monocytes were retained in the bone marrow and released upon treatment cessation, resulting in increased metastasis in mice [67].

Repolarizing TAMs towards an anti-tumoral state

Several strategies are currently investigated to repolarize TAMs into an anti-tumoral state. One of these strategies is using monoclonal antibodies against CD47, a protein commonly overexpressed by tumor cells, which binds to thrombospondin 1 and signal regulatory protein-α (SIRPα) on macrophages and inhibits phagocytosis. Strikingly, CD47 blockage allows for macrophage-mediated destruction of tumor cells [68]. Furthermore, agonists for toll-like receptors (TLRs) can be used to stimulate an anti-tumoral state in macrophages and were shown to have promising tumor-reducing effects [69].

Macrophages also express CD40, a member of the TNF receptor family, which when activated promotes a pro-inflammatory state of the cells. Agonistic anti-CD40 antibodies were found to induce MHC expression on macrophages and result in tumor regression in mouse models [70]. Another promising target is the MARCO receptor on macrophages, which when blocked leads to a switch in macrophage activation status and was shown to reduce tumor burden, especially when combined with checkpoint inhibitors [71]. Furthermore, inhibition of the myeloid-specific PI3Kγ pathway induced expression of MHC class II as well as of pro-inflammatory cytokines. At the same time, it reduced expression of Arg1, the enzyme responsible for conversion of L-arginine into ornithine and urea in TAMs. Importantly, low PI3Kγ activity correlated with enhanced patient survival [72].

Alike T cells, TAMs express programmed cell death protein 1 (PD-1) [73]. PD-1 is an example of an immune checkpoint receptor found on certain types of immune cells. Binding of its ligand PD-L1, which is commonly expressed by cancer cells, leads to immune tolerance and suppression of an anti-tumor immune response. Checkpoint blockade inhibitors are used to lift the negative regulation and instead promote an active anti-tumor immune response [74]. Therefore, checkpoint blockade inhibitors might also be useful in targeting TAMs and improve T cell-dependent immunotherapies [73].

1.1.5.3 TAMs in breast cancer

As for many other cancer types, high infiltration of TAMs in breast cancer correlates with poor prognosis [75]. In breast cancer, CD163 expression on TAMs was found to correlate with poor overall survival [76]. TAM infiltration correlates with a worse prognosis also in the TNBC subtype. Additionally, presence of proliferating macrophages was linked to HR negativity and basal-like cancer [77,78]. Furthermore, TAM infiltration was linked to higher chemoresistance in breast cancer [79].

In human breast cancer at least two distinct types of TAMs have been identified, a migratory subtype, that promotes metastasis and shows expression of MHC class II, and a sessile subtype, that is tumor-promoting and expresses CD206 on its surface. The migratory TAM subtype was described to be located in perivascular areas, whereas the sessile TAMs were found in hypoxic areas and at the tumor-stroma border [80]. Furthermore, a subtype of TAMs was identified which is associated with bone metastasis in breast cancer. These TAMs express high levels of CD204

and IL-4 receptor (IL4R). Upon blocking of IL4R outgrowth of bone metastasis was significantly reduced [81]. Additionally, recently a subset of TAMs was described to express high levels of podoplanin which supports the binding to lymphatic vessels and promotes lymphoinvasion in breast cancer [82]. Clearly, distinct subsets of TAMs exist in breast cancer and identification of their functions is necessary for the development of macrophage-targeted treatment strategies.

1.1.6 Natural killer cells

Like macrophages, natural killer (NK) cells belong to the innate immune system. However, their mode of action is entirely different. NK cells are able to recognize body-own infected, stressed or transformed cells and eliminate them without the need for prior priming, as it is the case for T and B lymphocytes of the adaptive immune system [83].

Compared to other cell types of the immune system, NK cells were discovered rather late, in 1975 [84]. How NK cells are able to distinguish between healthy and infected cells was proposed a few years later. It was concluded that NK cells must detect receptors on the surface of body-own cells which would inhibit them to get activated. These receptors were identified to be human leukocyte antigen (HLA) molecules. Loss or alteration of HLA expression upon infection or transformation of cells allows for the initiation of a NK cell response. This was termed the missing-self-hypothesis [85,86].

Nowadays, we know that NK cells display a multitude of activating and inhibiting receptors on their cell surface and NK cell activation is regulated by the balance of all incoming stimuli. Upon activation, NK cells degranulate and release granzyme B and perforin into the surroundings, damaging target cells and forcing them to undergo apoptosis [87]. NK cells can also release cytokines and chemokines, such as IFN-γ, tumor necrosis factor α (TNF-α) and CCL5, to stimulate an immunological response, recruiting additional immune cells to the site of infection [88].

Inhibitory NK cell receptors:

Inhibitory NK cell receptors are fundamental for the tolerance of healthy cells of the body. These types of receptors recognize HLA class I molecules that are present on the surface of all cells of the body. During their development NK cells undergo an ‘educational’ process and get eradicated if they falsely get activated by body-own cells to prevent the development of autoimmune diseases. A group of well-studied inhibitory receptors on NK cells are the killer-cell immunoglobulin-like receptors (KIRs) which recognize HLA-A, B and C molecules [89]. Another inhibitory receptor on NK cells consists of the heterodimer NKG2A/CD94 and specifically binds to HLA-E [90].

Activating NK cell receptors:

Activating NK cell receptors are essential for detecting unhealthy cells, such as stressed or infected cells, which often upregulate specific 'stress' ligands on their surface. One of the most studied activating receptors on NK cells is the NKG2D receptor. NKG2D signals as a homodimer and has eight different ligands which are all MHC class I related molecules. In humans these include MICA, MICB and UL binding protein 1-6 (ULBP1-6) [91]. Another group of major NK cell activating receptors comprises natural cytotoxic receptors (NCRs), NKp46 (NCR1), NKp44 (NCR2) and NKp30 (NCR3). NKG2C is one more example of an activating NK cell receptor. Competing with NKG2A it also binds to HLA-E, however with lower affinity [92]. The surface receptor CD16 on NK cells also acts as an activating receptor by binding the Fc part of antibodies and inducing antibody-dependent killing by NK cells [93]. Finally, besides inhibitory KIRs, also a group of activating KIRs can be found on NK cells [89].

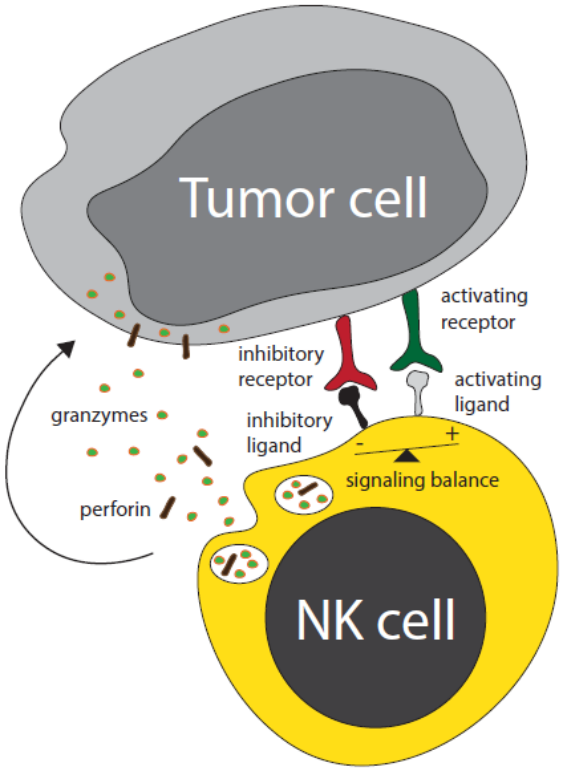

Figure 6. Interaction of NK cells with target cells

As mentioned previously, a fine balance of the stimulation of inhibitory and activating receptors determines the fate of the NK cells, either getting activated to fight or staying neutral and tolerate (see Figure 6) [94].

In humans, NK cells derive mainly from progenitors in the bone marrow and make up 10-15% of mononuclear cells in the blood. They are commonly defined as $CD3^{-}CD56^{+}$ and can be further divided by the extent of CD56 expression. The majority of NK cells is CD56 dim which concurs with greater ability to kill target cells and goes along with the expression of the surface

receptor CD16. CD56 bright NK cells are considered to be active cytokine producers and less efficient in target cell killing and show no or dim CD16 surface expression [95].

Circulating NK cells in the blood are usually in a resting state but can be activated by cytokines such as, interleukin 2 (IL-2) and interleukin 15 (IL-15), which are typically released at sites of infection or neoplastic growth. Activation by IL-2 or IL-15 promotes differentiation and proliferation of NK cells and enables production of perforin and granzyme B [96]. Even though IL-2 and IL-15 use similar IL2 receptor units, IL-15 has been shown to be a stronger activator of NK cells, rendering them more resistant to inhibition by oxidative stress [97,98].

Normally, upon activation NK cells are short lived. They perform their task, killing target cells, until they get exhausted and undergo apoptosis.

1.1.6.1 NK cells in cancer

As mentioned above, NK cells have the intrinsic ability to detect transformed cells [83]. They are therefore believed to exert an important role in immunosurveillance by detecting and eliminating tumor cells at an early stage. However, in the progression of tumor development they become overwhelmed by the immunosuppressive milieu. Interestingly, the NK cell population in non-small cell lung cancer was found to be enriched in cytokine producing CD56 bright NK cells and these NK cells were found to have a lower capacity of killing tumor cells [99]. Nonetheless, high NK cell infiltration has been associated with better clinical outcome in several types of cancer [100-102].

NK cell function is often compromised dramatically by tumor-promoting cell types. TAM or neutrophil derived arginase I-dependent depletion of L-arginine reduces NK cell proliferation and diminishes IFN-γ production by NK cells [103]. Furthermore, TAMs and MDSCs impair NK cell function by releasing immunosuppressive cytokines such as IL-10, prostaglandin E2 (PGE2) and TGF-β, which reduce NK cell activation and additionally attract more immune suppressive cell types like regulatory T cells (Tregs) [104]. Tregs are capable of directly inhibiting NK cells by surface expression of TGF-β which was found to induce downregulation of the NKG2D receptor on NK cells [105].

Furthermore, a regulatory NK cell phenotype has been identified. These regulatory NK cells were shown to release anti-inflammatory cytokines, IL-10 and IL-13, while also producing pro-inflammatory cytokines belonging to their normal repertoire (IFN-γ, TNF-α). These additional cytokines were sufficient to shift the balance and inhibit not only dendritic cells and macrophages, but also T cells [106,107]. The exact role of regulatory NK cells still needs to be precisely defined.

Tumor cells often present aberrant expression of NK cell receptor ligands on their surface. One example are the ligands for NKG2D that are commonly upregulated on transformed cells as an indication of stress and NKG2D has been shown to be an important mediator of cancer immunosurveillance [108]. Cancer cells, however, have developed ways to overcome this

mechanism by shedding NKG2D ligands. On one hand, this prevents NK cell killing as no activating ligand is detected. On the other hand, it also impairs NKG2D expression on NK and T cells as a result of continuous receptor stimulation [109].

In addition, MHC class I receptors are often downregulated by cancer cells to overcome immunosurveillance by T cells [110]. Even though, theoretically, NK cells should get activated upon recognizing these tumor cells they were found to exist in an anergic state in MHC class I-deficient tumors [111], proving the effective strategies of cancer cells to overcome immune surveillance.

NK cells have recently moved into the spotlight of attention for their use as immunotherapies of different cancers. Especially different types of hematopoietic cancers benefited from NK cell-based immunotherapies [112,113].

1.1.6.2 NK cells in breast cancer

As mentioned previously, infiltration of NK cells into the tumor microenvironment correlates with better survival in breast cancer patients [100]. Furthermore, breast cancer patients with high expression of NKG2D ligands, MICA/B and ULBP2, had improved outcome with increased relapse-free periods [114]. However, natural killer activity of peripheral blood mononuclear cells was significantly reduced in patients with breast cancer, suggesting suppressed NK cell function in cancer [115].

Nonetheless, NK cells are important contributors to current treatment strategies for breast cancer. NK cells were shown to infiltrate the TME after treatment with HER2-targeted therapies. Actually, these NK cells actively contribute to the anti-tumor effects of the HER2 antibody treatment through their ability of antibody-mediated cellular cytotoxicity (ADCC) [116]. In TNBC, an antibody targeting epidermal growth factor receptor (EGFR), which is often upregulated in basal breast cancer, has been described to elicit ADCC mediated by NK cells [117]. Taken together, NK cells as well as NK cell ligands on cancer cells play a critical role in immune evasion and treatment strategies in breast cancer.

1.1.7 Inflammation and cancer

In 1863, the German physician Rudolf Virchow noted infiltration of leukocytes into the tumor site and made a first connection between cancer and inflammation [118]. Since then the consequences of inflammatory processes have been described in detail, from the initial steps of tumor development up to its influence on metastatic growth. Importantly though, several scenarios have to be distinguished in the context of cancer inflammation [119].

Acute inflammation

Acute inflammation is the normal reaction of the body's immune system to a potential threat to the body. Immune cells get recruited to the site which produce inflammatory cytokines and release ROS into the environment to kill the intruder. In case of cancer, acute inflammation is a desired process. Activated immune cells have the ability to recognize and kill cancer cells and potentially resolve the issue. In fact, most pre-cancerous lesions are detected by the immune system and eliminated before any damage has been made to the body, which has been termed cancer immunosurveillance [120].

Extrinsic chronic inflammation

If the immune system fails to clear the initial threat this can lead to chronic inflammation, which leaves a constantly activated immune system localized to a specific organ/site. The continuous release of inflammatory signals as well as factors such as ROS can result in damage to the tissue and induce mutations in surrounding cells. Furthermore, the release of growth signals can promote abnormal cellular growth. There are several examples where chronic inflammation is linked to cancer development. Infection with Helicobacter pylori has been correlated with increased risk of developing gastric cancer [121]. Human papilloma virus (HPV) has been linked to cervical cancer and viruses of the hepatitis family can cause hepatocellular carcinomas [122,123]. As these examples indicate, extrinsic chronic inflammation is caused by factors extrinsic to the cancer cells.

Intrinsic chronic inflammation

Intrinsic chronic inflammation, on the other hand, indicates that the stimulus underlying the chronic inflammation originates from the cancer cell itself. During the process of a cell becoming cancerogenic mutations occur that can affect the interaction with the immune system, such as oncogene-driven production of inflammatory signals. This continuous release of inflammatory factors leads to the recruitment and infiltration of immune cells and the establishment of a chronic inflammation [124].

The concept of immunoediting includes three stages of tumor-immune system interaction. Elimination, which involves acute inflammation and the potential complete eradication of the tumor. A state of equilibrium, where the immune system is fighting the cancer, but the tumor cells are continuously proliferating and mutating. And finally, escape, where some clones manage to evade the detection of the immune system and start to edit the immune system for its benefits [120].

Chronic inflammation, either intrinsic or extrinsic, is considered favorable for tumor growth due to the continuous selection of tumor cell clones able to escape recognition and elimination by the immune system. Additionally, as discussed above in the chapter 'The Tumor Stroma',

immune cells recruited into the TME are educated by the tumor to support it with growth factors, prevent it from being attacked by the immune system and facilitate invasion and metastasis [44]. Currently, researchers are investigating strategies to shift the inflammatory profile away from a chronic and more to an acute stage, where immune cells are released from their inhibitions and are able to exert their anti-tumoral potential. Immunotherapies, such as checkpoint therapies, are developed on this underlying principle [125].

Intriguingly, TAMs are firmly engaged in the relationship between cancer and inflammation. By secreting pro-tumoral cytokines they are actively shaping an immunosuppressive environment [59].

1.1.8 The NF-κB Pathway

The NF-κB pathway is known as the master regulator of inflammation since it is involved in the regulation of almost all processes related to inflammation. This pathway comprises a key family of transcription factors, which can form several different homo- and heterodimers. The NF-κB family consists of p65 (RELA), p105 /p50 (NFkB1), p100/p52 (NFkB2), RelB (RELB) and c-Rel (REL).

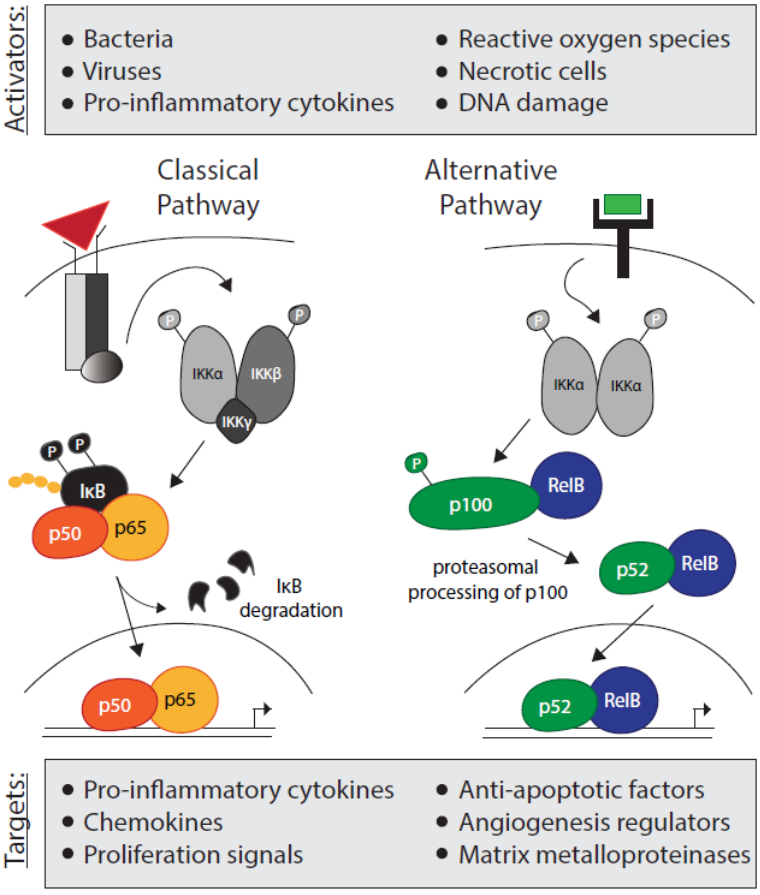

Figure 7. The NF-κB pathway, its activators and targets.

A multitude of stimuli can lead to the activation of the NF-κB pathway that can be divided into a classical (canonical) and an alternative (noncanonical) pathway (see Figure 7). Stimuli include viral and bacterial proteins, DNA damage, oxidative-stress, necrotic bi-products, or pro-inflammatory cytokines. The most common ligands for the classical NF-κB pathway are LPS, TNF-α and IL-1. Upon binding of the ligand to the receptor the IKK kinase complex gets phosphorylated and in turn induces the phosphorylation of IκB, which then gets ubiquitinated and degraded. IκB is usually bound to p65 and retains it in the cytoplasm of the cells. However, upon its degradation p65/p50 is released and can translocate into the nucleus where it binds to its target genes and initiate their transcription [126].

The alternative pathway, on the other hand, gets activated by specific ligands that belong to the TNF superfamily such as CD40 and RANKL. The alternative pathway acts slower than the classical pathway and mainly relies on p52/RelB heterodimers for transcriptional activity [127].

Depending on the stimulus and on its binding partners NF-κB can activate the expression of different groups of target genes, which can belong to cytokines and chemokines, proliferation signals, anti-apoptotic factors, angiogenic regulators or matrix metalloproteinases [128].

1.1.8.1 NF-κB in cancer

The NF-κB pathway plays a bit of a dual role in cancer. On one hand, it is fundamental for pro-inflammatory processes and essential for anti-tumoral functions of immune cells [129,130]. On the other hand, NF-κB activation in cancer cells promotes their survival and proliferation, while suppressing apoptosis. Besides that, NF-κB induces chronic inflammation promoting a tumor-supporting microenvironment and has been reported to influence epithelial-to-mesenchymal transition (EMT), invasion and metastasis, as well as angiogenesis. It has also been found to contribute to therapy resistance. For these reasons, it is well established that activation of the NF-κB pathway is heavily involved in cancer development and progression [131].

1.1.9 Hypoxia

Hypoxia is defined as reduced oxygen levels compared to the regular state. Oxygen levels vary greatly within the body, however, on average physiological oxygen levels in tissues lie around five percent [132]. Hypoxic regions can be found in most solid tumors due to rapid and uncontrolled cell growth. Inflammation also contributes to hypoxia because of increased metabolic activity and infiltration of immune cells that contribute to increased cellular density in the tissue [133].

Under normal conditions, e.g. the presence of sufficient oxygen, oxygen sensing prolyl hydroxylases (PHDs) drive the hydroxylation of a group of hypoxia inducible factors (HIF-1α, HIF-2α and HIF-3α; in this thesis the focus will be on HIF-1α). This hydroxylation allows then binding of the von Hippel-Lindau (VHL) protein to HIF-1α which consequently results in the polyubiquitination of HIF-1α by an E3 ligase complex that consists of Rbx1, Cullin 2, Elongin

B and Elongin C (ECV complex) [134]. The polyubiquitination by the ECV complex leaves HIF-1α as a target for rapid degradation by the proteasome (see Figure 8) [135].

In case of a hypoxic environment the PHD enzymes cannot exert their function, which results in increased stability of HIF-1α and can therefore enter the nucleus where it dimerizes with its co-factor HIF-1β, binds hypoxia responsive elements (HREs) and activates transcription of its target genes.

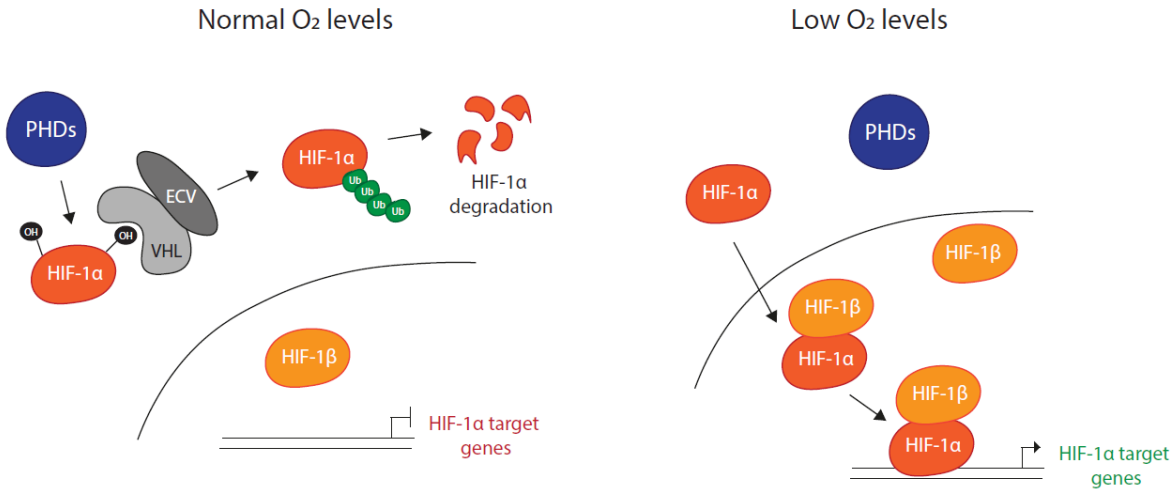

Figure 8. The HIF-1α pathway under normal or low oxygen conditions.

HIF-1α acts as a major transcription factor regulating numerous genes involved in angiogenesis, such as VEGF. Besides that, HIF-1α has been reported to transcribe genes relevant in EMT, metastasis and chemo- and radio-resistance [136]. Hypoxia also exerts an impact on immune cells in the tumor microenvironment, promoting the suppressive functions of tumor associated macrophages and regulatory T cells [137,138]. Interestingly, hypoxia also affects inflammation and several reports highlight a dynamic crosstalk between the hypoxia pathway and the NF-κB pathway [139-141]. Importantly, hypoxia and hypoxic gene expression is usually associated with poor patient survival [142].

1.1.10 Angiogenesis

Unavoidably, the continuous, uncontrolled growth of tumor cells reaches a point where oxygen and nutrition levels are insufficient for cellular survival and where waste products cannot be cleared from the TME. As discussed in the previous section (‘Hypoxia’), low oxygen levels drive the expression of an adaptational cellular program to overcome these obstacles by inducing angiogenesis. This point is referred to as angiogenic switch and is essential for advanced tumor growth [143]. Angiogenesis is the process of blood vessel formation by endothelial cells. Blood vessels are comprised of endothelial cells and covered by pericytes, which are stromal cells that give structural support to the vessels. Upon pro-angiogenic cues

like VEGFA (or VEGFC, which induces lymph vessel formation), VEGFR2 gets activated on endothelial tip cells which induces sprouting and proliferation of the cells. Endothelial tip cells migrate towards the cytokine gradient followed by stalk cells extending the sprout and forming the lumen. Platelet-derived growth factor B (PDGFB) is expressed by sprouting endothelial cells and leads to the recruitment of pericytes to stabilize vessel formation. Sprouting and branching of the vessels continues until an organized network has been constructed to support the tissue with sufficient oxygen [144].

In cancer, angiogenesis is needed for oxygen supply and the transportation of nutrients to as well as waste products from the tumor [143]. However, unlike angiogenesis in healthy tissues, in tumors this process is less strictly regulated resulting in unorganized and leaky blood vessel formation. This prevalent leakiness increases vessel permeability and the ease of cell migration, hence, increasing the metastatic potential of the tumor [145]. As mentioned earlier (see section 'Tumor associated macrophages'), TAMs play a significant role in tumor angiogenesis. TAMs as well as tumor cells in hypoxic areas induce the expression of VEGF which drives the process of angiogenesis. Furthermore, by expressing MMPs, TAMs are able to remodel the extracellular matrix to create space for vessel formation [146].

Angiogenesis correlates with poor survival in many cancer types and multiple strategies to target this process have therefore been investigated [147-149]. Several drugs have been approved as anti-angiogenic therapy, most of them targeting members of the VEGF family. However, side effects and the fact that tumor cells manage to become resistant and escape therapeutic intervention has so far limited feasible treatment strategies [150].

1.1.11 Multidrug Resistance in Cancer

There are plenty of drugs against cancer available on the market and often they show tumor-reducing effects at first. However, tumors are extremely heterogeneous. Clonal heterogeneity exists intra-tumoral, within the primary tumor, as well as between primary tumor and metastatic lesions, due to a constant turnover and mutational load, but it is also caused by therapeutic pressure [151]. Often a single drug is effectively killing most of the tumor cells resulting in remission. However, a few clones might be able to survive the treatment and allow regrowth of the tumor [152]. To overcome single drug resistances physicians started treating cancer patients with combinations of chemotherapies with different modes of action, which helped to reduce drug resistances. Even though combinational therapies showed greater effects than single treatments efficiency plateaued at some point. Also, against more recent cancer therapies, such as targeted therapies and immune checkpoint therapies, cancer cells have been shown to develop resistance [153].

In case of chemotherapy tumor cells have been described to utilize different ways to evade killing. One frequent mechanism involves the upregulation of specific proteins located in the cell membrane that act as transporters for various molecules to pump them out of the cell. The largest group of these transporters is the ATP-binding cassette (ABC) family, many of which

members are often found to be upregulated in different cancer types, including breast cancer [154,155]. (This will be discussed in more detail in the results and discussion part of Paper IV).

Strikingly, it seems tumor cells have an extreme ability to adapt, in order to ensure their survival. It is therefore a huge challenge to overcome multidrug resistance in cancer, which, if ever resolved, will save millions of lives.

1.2 P73

1.2.1 Part of the p53 Family

TP73 is a gene that is part of the p53 family together with TP53 and TP63. TP53 is the most studied tumor suppressor gene existing to date [10]. The proteins of p53, p63 and p73 function as transcription factors for a variety of target genes including players fundamental in the regulation of cell cycle arrest and apoptosis such as p21 and Bax, as well as MDM2 that is the main negative regulator of p53 stability [156]. Activation of the members of the p53 family can be induced by multiple internal and external stimuli. These include DNA damage, hypoxia, NTP depletion and oncogene activation among others [157]. All members of the p53 family share both structural and functional homology. In general, the protein structure contains a transactivation domain (TAD), a DNA binding domain (DBD) as well as an oligomerization domain (OD). All three family members contain proline rich sequences (PR), but only the p63 and the p73 gene contain a sterile alpha motive domain (SAM) at the C terminus (see Figure 9). Proteins of the p53 family can form tetramers of homo-complexes, in the case of p53, or hetero-complexes, p63 and p73, and can bind to the same DNA sequences since they share 100% homology in the residues interacting directly with DNA [158,159].

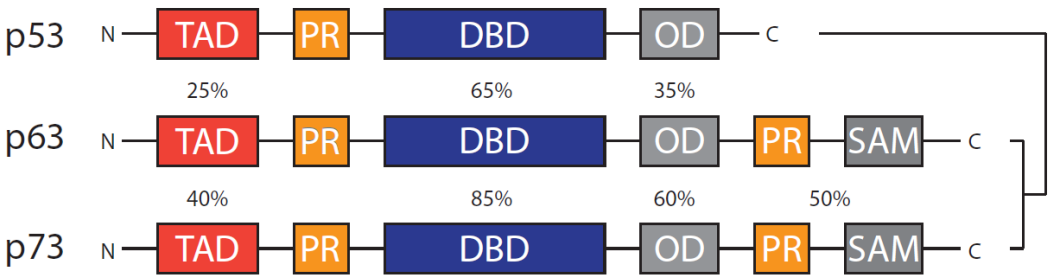

Figure 9. Structural similarities within the p53 protein family.
(Percentages indicate structural homology with p53.)

The cellular availability of p53 family members is tightly regulated at a post-translational level by ubiquitination. MDM2 is the best studied E3 ubiquitin ligase found to modulate the transcriptional activity of p53, p63 and p73 [160]. Due to its crucial regulation of p53 activity, MDM2 has been termed the gatekeeper of p53. Under normal conditions MDM2 binds to p53 and ubiquitinates it, which leads to p53's proteasomal degradation. Furthermore, MDM2 can also bind to the TAD of all proteins of the p53 family, thereby inhibiting their transcriptional activity [161]. On top of that, a recent study suggests the involvement of MDM2 in ubiquitination and proteasomal degradation of p73 [162]. Upon cellular stress p53 gets phosphorylated at Ser^{15} by stress-induced kinases (ATM, ATR, c-Abl), which disables binding of MDM2, thus allowing for protein stability [163]. Additionally, MDM2 levels are reduced upon stress signals which releases the inhibition on p53 and the other family members [164]. In turn, MDM2 expression was shown to be partly regulated by p53 family members, indicating an autoregulatory feedback loop on p53 activation [165]. An important regulator of p73 proteins is

the ubiquitin-protein ligase Itch. P73, as well as p63, have been reported to bind Itch, which leads to their proteasomal degradation [166,167]. Upon DNA damage, Itch is downregulated which lifts Itch-mediated degradation and allows for accumulation of p73 and p63 proteins. Opposingly, YAP-1 (Yes-associated protein 1) binds the same region of p73 as Itch and thereby interferes with Itch-dependent p73 degradation [168]. While so far it is unknown if YAP-1 also effects p63, p53 was shown to be unaffected.

Even though several common target genes of the p53 family have been confirmed, the individual members were found to play unique roles in regulating various processes during embryonic as well as tumor development [169,170]. These differences are thought to be due to distinct activation and association with specific binding partners required for regulating transcription.

P53

The TP53 gene has been described the first time in 1979 and since then has become one of the most studied genes in cancer [171]. This is due to the fact that the TP53 gene is found mutated in around thirty to fifty percent of all cancers, being the most frequently mutated gene so far described in the cancer genome [172]. When not functionally inactivated by mutations, some mutant p53 variants were found to display gain-of-function properties [173]. Actually, when p53 was discovered it was thought to be an oncogene and it took the scientific community several years to agree upon p53 being a tumor suppressor [174]. Since then p53 is commonly referred to as 'the guardian of the genome' being able to induce cell cycle arrest, DNA repair or apoptosis in response to potential oncogenic events [175].

Importantly, several viral proteins have the ability to interfere with the functions of wildtype p53, including the Simian Vacuolating Virus 40 (SV40) large T-antigen protein, which was shown to bind p53 and thereby prevent p53-mediated transcription of pro-apoptotic genes [176]. Additionally, the E6 protein, which is found in HPV, is able to bind p53 and promotes its proteasomal degradation by an E3 ligase [177]. Furthermore, also E7, another viral protein of HPV, has been described to interfere with p53-dependent cell cycle control [178]. Yet, another mechanism to inhibit p53-mediated transcription has been described for the mouse polyoma virus. Polyoma virus proteins seem to interfere with p53 activation by disrupting upstream ARF signaling [179].

In line with the frequent inactivation of p53 in cancers, p53 knockout mice were shown to be prone to develop lymphomas and sarcomas at around six months of age. However, most animals deficient for p53 do not show any indication of developmental deficiencies [180]. (Of note, a small percentage of females were found to develop exencephaly [181].)

P63

Contrary to p53 knockout mice, mice deficient for p63 die within a day from birth, showing evident malformations. They display severe defects in the establishment of stratified epithelium which causes abnormalities in limb and craniofacial development [182]. These findings revealed an important role for p63 in the maintenance of epithelial precursor cells.

Besides its function in epidermal regulation, p63 has also been described to play a role in progression of various cancers, including hepatocellular carcinoma and squamous cell carcinoma [183-186]. TP63 and TP73 share similar gene structures, leading to various tumor-suppressing and oncogenic protein isoforms with opposing functions on tumor development (see following section) [187].

P73

P73 was first discovered in 1997, almost 20 years after its family member p53. The TP73 gene is located on the chromosome 1p36 and is thought be expressed mono-allelic [188]. This region of chromosome 1 is often lost in cancer, including neuroblastoma [189].

TP73 can be transcribed from two distinct promoters [190,191]. Transcription from the proximal P1 promoter gives rise to the full-length protein which acts as a tumor suppressor in a similar fashion as p53. This full-length protein is called TAp73 since it contains the transactivation domain. Transcription from an alternative internal P2 promoter, however, leads to a N-terminally truncated version of p73 that is missing the transactivation domain. This protein has been termed ΔNp73 and acts as a dominant negative. To increase complexity, alternative splicing of the pre-mRNA leads to various additional C-terminal isoforms, termed α-η, and the splice variants p73ΔEx2 and p73ΔEx2/3 in the N-terminal region (see Figure 8) [192].

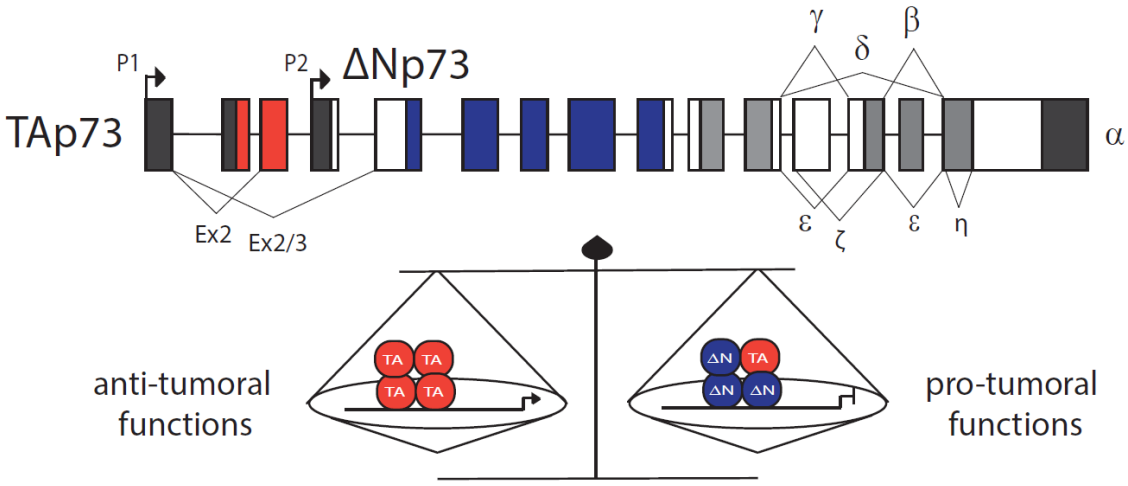

Figure 10. TP73's gene structure and the balance between its isoforms.

P73 deficient mice are viable but show eminent mortality rates at young age. These mice present severe defects in the neurological development of the central nervous system as well as hypoplasia of the olfactory bulb and 100% penetrance of severe hydrocephalus [193,194]. Additionally, p73 knockout mice present severe chronic airway infections [194]. Surprisingly, neither p63 nor p73 knockout mice show a clear predisposition for spontaneous tumor development. However, mice heterozygous for both p63 and p73 but with wild type p53 exhibit reduced survival due to the development of various tumor types manifesting the importance of functional p63 and p73 in tumor development independent of p53 [195].

Isoform specific knockout mice were needed to investigate the impact of TAp73 and ΔNp73 individually. Mice deficient for TAp73 present defects in hippocampal development as well as in maintenance of genomic stability. Furthermore, these mice display chronic respiratory tract infections, which were discovered to be due to severe defects in the process of multiciliogenesis. Actually, recently TAp73 was shown to play a profound role as transcriptional regulator in multiciliated cells [196,197]. TAp73 knockout mice are infertile and show an increased rate of spontaneous as well as carcinogen-induced tumor development [198]. These findings confirm the importance of TAp73 as a tumor suppressor.

In contrast, ΔNp73 knockout mice are fertile and show a normal life span but present some degree of neurodegeneration. $\Delta Np73^{-/-}$ cells exhibit increased p53 target gene expression at steady state levels and even higher after DNA damage which confirms the notion that ΔNp73 usually inhibits the function of p53. As could be suspected, transformed $\Delta Np73^{-/-}$ cells show a drastically reduced ability to form tumors upon injection into immunocompromised recipient mice [199]. Hence, this furthermore confirms ΔNp73s action as an oncogene. Taken together, these findings highlight the importance of studying specific p73 isoforms rather than looking at total p73 levels.

1.2.2 P73 in Cancer

Interestingly, TP73 is rarely mutated in cancer. Instead it is thought that there is a shift in balance between the expression of the two isoform groups, TAp73 and ΔNp73 [200]. This leaves p73 as an exception to the standard Knudson tumor suppressor, since TAp73 gene expression does not have to be lost in order to overcome its tumor suppressing functions. Since ΔNp73 binds the same DNA binding motifs as TAp73, p53 and p63 it can compete for binding to the DNA and thereby inhibit transcription. Furthermore, it can form hetero complexes with TAp73 but also with p63 and thereby actively inhibit their function [190,201]. Additionally, studies have revealed that wild type and mutant p53 can bind p73 after JNK-mediated phosphorylation of Thr^{81} in the DBD of p53. This leads to structural changes that increase affinity, thereby enhancing wildtype p53's function of promoting pro-apoptotic gene expression [202]. Consequently, it seems that cell fate is dependent on the delicate balance and the complex interactions of all the members of the p53 family.

The significance of p73 expression in tumor tissue has been validated in several studies using patient data with the conclusion that upregulation of total p73 protein is correlated with poor prognosis in various cancers including colorectal adenocarcinoma, hepatocellular carcinoma and lung cancer [203-205]. However, when looking at specific isoforms, ΔNp73 can be found upregulated in multiple types of carcinoma and is correlated with poor prognosis in neuroblastic tumors, colon and breast cancer as well as gynecological cancers [201,206-208]. Intriguingly, in many cancers both TAp73 and ΔNp73 are upregulated, however, the outcome seems to depend on the balance between the isoforms [209]. In contrast to carcinomas, in a number of leukemias and lymphomas the P1 promoter, responsible for expression of the full-length isoform TAp73, is hypermethylated which results in inhibition of expression and, consequently, loss of TAp73 function [210].

Importantly, increased TAp73 expression has been described to be induced by chemotherapeutic drugs in p53 mutant tumor cells. Concurrently, TAp73 inhibition resulted in chemo-resistance in tumor cells regardless of their p53 status [211]. Overall, it can be concluded that p73 has been identified as a promising biomarker for prognosis of cancer patients especially when dissecting the individual isoforms.

1.2.2.1 P73 in Breast Cancer

Both tumor-suppressing and tumor-promoting p73 isoforms are commonly found to be upregulated in breast cancer. However, only upregulation of ΔNp73 has been found to correlate with worse prognosis in breast cancer patients [207]. TP73 expression can be regulated by epigenetic changes in promoter methylation. A recent study describes the frequent methylation of CpG islands in the TP73 promoter in breast cancer with varying intensity within different molecular subtypes. Within this study, the expression of ΔNp73 was found to correlate with higher histological grade of the tumor and showed a trend towards worse overall survival in invasive ductal carcinoma [212]. The DNA demethylation agent 5-aza-2'-deoxycytidine was found to promote upregulation of TAp73, while downregulating ΔNp73, and thereby resulting in enhanced cell cycle arrest and induction of apoptosis [213].

The inactivation of p53 in various breast cancer cell lines led to the increased expression of TAp73 mediated through the transcription factor E2F-1, indicating an intrinsic rescue mechanism in the cells [214]. Furthermore, in p53 deficient breast cancer cell lines TAp73 was shown to take over induction of cell cycle arrest and apoptosis in response to chemotherapeutic drugs [215].

Overall, it can be concluded that p73 isoforms seem to play a significant role in the progression and the therapeutic response in breast cancer.

1.2.2.2 P73 in Melanoma

While in other cancers ΔNp73 is often upregulated and associated with worse survival, in case of melanoma ΔNp73 levels were described to be unchanged. Instead the oncogenic p73 member p73Δex2/3 was described to be upregulated in melanoma and especially in invasive melanoma [216]. This isoform is transcribed from the P1 promotor of the TP73 gene, but due to alternative splicing in exon 2 and 3 it is missing the transactivation domain and consequently acts as a dominant negative, comparable to ΔNp73 [217]. In melanoma the simultaneous upregulation of p73Δex2/3 and TAp73 was detected to be regulated by E2F-1-mediated transcription from the P1 promoter [216]. Furthermore, p73Δex2/3 was found to promote the expression of genes involved in stemness and depletion of p73Δex2/3 reduced metastasis in melanoma xenograft models [218].

2 AIMS OF THE THESIS

The overall aim of this thesis is to understand the specific functions different p73 isoforms exert in regulating the tumor microenvironment, with a strong focus on breast cancer.

Specific aims of the studies included in this thesis:

Study I: To investigate how TAp73 influences macrophage infiltration and phenotype in breast cancer.

Study II: To get an insight if ΔNp73 is involved in the regulation of NK cell ligands and its effects in breast cancer.

Study III: To unravel the role of ΔNp73 in hypoxia regulation – using breast cancer as a model.

Study IV: To determine the effects of ΔNp73 on multidrug resistance in breast cancer and melanoma.

3 RESULTS AND DISCUSSION

3.1 PAPER I

TAp73 represses NF-κB-mediated recruitment of tumor-associated macrophages in breast cancer.

Up to date there is little information if and how different p73 isoforms regulate immune cells in cancer. In a recent publication we identified TAp73 as an important regulator of pro-angiogenic factors in breast cancer [219]. Many of these angiogenic activators are also involved in the recruitment of immune cells. We were therefore curious to investigate if TAp73 plays a role in immune cell recruitment in breast cancer.

To follow up on our previous findings, we used the publicly available TCGA breast cancer data set and compared TAp73 low and high expressing samples, while removing all samples showing expression of the oncogenic isoform, ΔNp73, to exclude possible reciprocal interactions. Gene set enrichment analysis and consequent pathway analysis displayed a substantial correlation between low TAp73 expression and a strong inflammatory signature in breast cancer biopsies. Furthermore, several gene sets enriched in low TAp73 samples were tightly linked to an increased NF-κB signature. Using TAp73 deficient cells we observed that loss of TAp73 resulted in higher activity of the NF-κB pathway, confirming our observations in human breast cancer patients.

Inflammation and an active NF-κB pathway are strongly linked to tumor progression and metastasis, as described in the chapters 'Inflammation in cancer' and 'NF-κB in cancer'. While not so much information is available for different p73 isoforms, several studies describe the interaction of p53 and the NF-κB pathway. P53 was found to repress the NF-κB pathway and concomitant pro-inflammatory cytokine release by interfering with IκB degradation via diminished proteasome activity [220,221]. In contrast, accumulation of mutant p53 was found to correlate with increased NF-κB activation in human colon cancer [222]. Mutant p53 was furthermore shown to induce transcription of NF-κB2, supporting proliferation and motility of the cells [223]. Clearly, there is a bilateral interplay between NF-κB and p53, and depending on the ratio, this either results in NF-κB inhibition by p53 or otherwise inhibition of p53 by members of the NF-κB pathway. NF-κB and p53 were shown to compete for the same transcriptional co-activator, p300/CBP (CREB-binding protein) [224-226]. Also p63 was shown to interact with p300/CBP to regulate transcription of target genes, such as p21 [227].

Intriguingly, just like the other family members, p73 isoforms were found to interplay with the NF-κB pathway. IkappaB kinase beta (IKKβ) has been described to promote cell survival through inhibiting p53 functions by phosphorylating and stabilizing ΔNp73α [228]. Furthermore, TAp73 has been found to compete with the NF-κB subunit p65 for binding to p300/CBP [229,230].

This reciprocal competition for the transcriptional co-activator might be the underlying mechanism how TAp73 inhibits the expression of the NF-κB-dependent production of pro-inflammatory cytokines that we describe here.

To validate the upregulation of proinflammatory factors detected in TAp73 deficient tumors we analyzed mRNA expression and secretion levels of several cytokines and chemokines in wildtype and TAp73 knockout cells. This left us with CCL2 showing the highest upregulation both on mRNA levels as well as in protein secretion in TAp73 deficient cells. We confirmed the downregulation of CCL2 upon overexpression of TAp73 in several murine and human breast cancer cell lines. Interestingly, the TAp73β isoform, but not TAp73α, led to a reduction in CCL2 levels. Several studies have shown the different effects TAp73α and TAp73β have on target gene expression, with TAp73β comprising stronger transcriptional activity [231]. It is suggested that this stems from their structural differences as TAp73α contains a SAM domain in its C-terminal region [232]. This SAM domain has been shown to exert inhibitory functions on TAp73α ability to transcribe target genes. In line with this, the C-terminal region, including the SAM domain, has been described to inhibit interaction with the co-transcriptional activator p300/CBP, thereby suppressing transcription of target genes [233].

While overexpression of TAp73 repressed CCL2 levels, knockdown of TAp73 using siRNAs resulted in an upregulation of CCL2 in human breast cancer cell lines. To show that TAp73-mediated CCL2 inhibition is NF-κB dependent, we used two small molecule inhibitors targeting different stages of the NF-κB pathway. Inhibition of the subunit IKKβ or specifically inhibiting p65 resulted in a strong reduction of Ccl2 levels in MEFs deficient for TAp73, normalizing Ccl2 to wildtype levels. This demonstrates that the increased expression of Ccl2 in TAp73 deficient cells is due to NF-κB activity. Additionally, combined deletion of the two distal NF-κB binding sites in the Ccl2 promoter, that are known to be important for Ccl2 expression, resulted in complete inhibition of Ccl2 promoter activity. Adding TAp73β did not lead to further reduction, suggesting that these binding sites are essential for Ccl2 promoter activity, and that TAp73β suppresses NF-κB-dependent transcriptional activation of the Ccl2 promoter.

Since CCL2 is one of the main chemokines involved in macrophage recruitment, we were intrigued to investigate the influence that loss of TAp73 in the tumor cells might have on macrophages in the tumor microenvironment. In general, not much is known about the influence of p73 on macrophages. To date, the only link between p73 and macrophages is a study investigating the loss of TAp73 in macrophages in mice. The results indicate that TAp73 is needed for macrophage activation and innate immunity. A lethal challenge with LPS resulted in a more pro-inflammatory activation state of the TAp73$^{-/-}$ macrophages compared to wildtype, indicated by higher expression of MHC class II and a decrease in phagocytosis. Furthermore, TAp73$^{-/-}$ mice showed higher levels of pro-inflammatory cytokines in the blood, such as TNF-α, IL-6 and IL-1β [234].

In the case of its family member p53 some more data is available describing its influence on macrophages in cancer. A recent study by Cooks et al. elucidates the pathway of how mutant

p53 is able to reprogram macrophages in the tumor microenvironment to acquire a tumor supporting phenotype. There they describe how colon cancer cells with mutant p53 release exosomes containing miR-1246. Thereafter, these exosomes are ingested by macrophages leading to an alteration in their phenotype, which ultimately has an impact on tumor growth [235]. Furthermore, several studies indicate a role for p53 in the regulation of CCL2. In glioblastoma mutant p53 correlated with NF-κB-dependent upregulation of CCL2 [236]. Furthermore, in a murine model for ovarian high-grade serous carcinoma loss of p53 led to increased levels of CCL2 and consequently an elevation of infiltrating immunosuppressive myeloid cells [237].

Up to date, the engagement of TAp73 in TAM recruitment and activation state in cancer has been entirely unknown. To study the effects of TAp73 loss on TAMs we used two murine *in vivo* models. The first one being a subcutaneous model where we utilized wildtype or TAp73 deficient transformed MEFs to study tumor growth and the TME. The second model represented a more disease relevant model for modelling breast cancer. We utilized the MMTV-PyMT model (on a C57Bl/6 background) which develops spontaneous tumors in the mammary gland in mice due to the expression of the polyoma virus middle T oncoprotein under the mouse mammary tumor virus LTR, which restricts tumor development to the mammary epithelium [238]. These mice were crossed with TAp73 wildtype or knockout mice and were monitored for spontaneous tumor formation. From these tumors cell lines were prepared. The cell lines were validated for Ccl2 expression. TAp73 knockout showed increased Ccl2 expression and secretion compared to wildtype cells. Cells were then injected orthotopically into the mammary gland fat pad of wildtype C57Bl/6 mice. This strategy allowed us to specifically investigate the effects of loss of TAp73 in tumor cells on the TME, while all other cell types were wildtype for TAp73.

Using these models, we show that loss of TAp73 leads to increased infiltration of TAMs in breast cancer. The TAMs were found to present enhanced surface expression of CD206 and CD204, indicating a tumor-promoting activation state of the cells. Additionally, we analyzed human breast cancer biopsies for TAp73 expression levels and compared those with TAM infiltration and activation. Low expression of TAp73 was found to correlate with higher levels of CD68 and CD163, markers commonly used for general macrophages or pro-tumoral macrophages, respectively. Importantly, a recent study found that high infiltration of CD163 positive macrophages correlated with poor prognosis in TNBC, furthermore strengthening the importance of our findings [239].

Interestingly, TAMs are known to influence the behavior of mouse breast cancer cells through the secretion of TNF-α which induces enhanced NF-κB activity in the cancer cells and enhances their invasive potential [240]. Increased macrophage infiltration due to an activated NF-κB pathway in TAp73 deficient tumors and the concurrent increase in TNF-α secretion could theoretically further promote NF-κB activity in the cancer cells, resulting in a tumor-promoting circuit.

Taken together, here we identify TAp73 as an important regulator of NF-κB-mediated expression of pro-inflammatory cytokines as well as of macrophage recruitment and activation in breast cancer (see Figure 9). If TAp73 has this function also in other types of cancer remains to be investigated. Considering that TAp73-dependent regulation of the NF-κB pathway was not only observed in breast cancer cell lines, but also in transformed MEFs, which were used for mechanistic studies, indicates that this mechanism might not be unique to breast cancer. Furthermore, coherent with our own findings, loss of TAp73 in macrophages was found to increase secretion of pro-inflammatory cytokines [234]. Despite macrophages being completely different from tumor cells, TAp73 knockout results in the same outcome in both cell types, namely an increase of pro-inflammatory factors, pointing to a general role for TAp73 in the regulation of NF-κB.

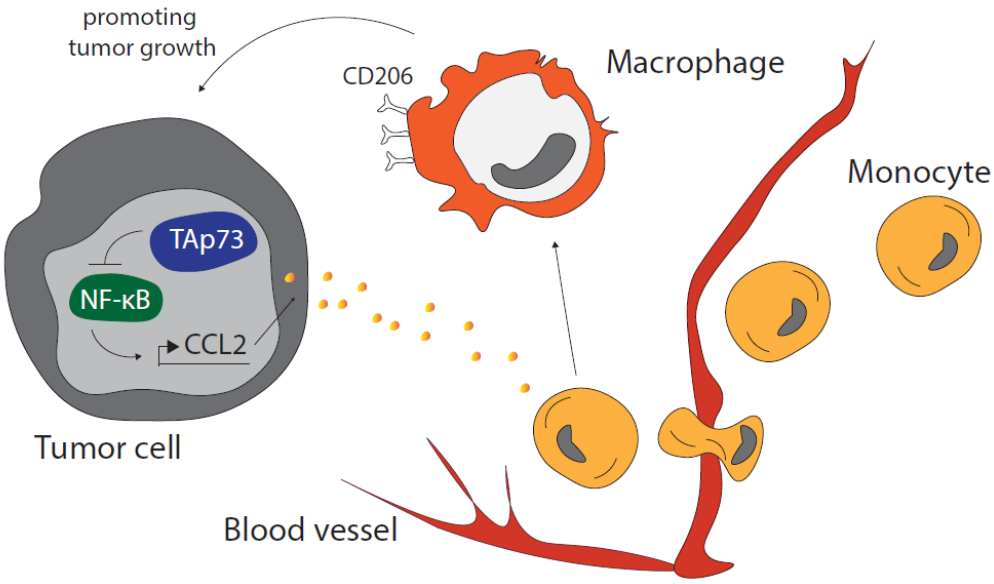

Figure 11. The effects of TAp73 on NF-κB regulation and TAM recruitment.

Many different types of cancer are known to have an increased activation of the NF-κB pathway as well as high macrophage infiltration, both factors exerting general tumor-promoting effects [241,242]. It might be that TAp73 plays a significant role in regulating tumor progression by inhibiting NF-κB and associated tumor-promoting macrophage infiltration, and that tumors that manage to overcome this inhibition gain growth advantage through an increase in proliferation signals and support by macrophages. TAp73-mediated inhibition of the NF-κB pathway might present a general mechanism true for many cancer types.

3.2 PAPER II

Loss of ΔNp73 leads to an increase in NKG2D ligand expression, while not affecting NK cell-mediated tumor cell killing in breast cancer.

NK cells serve as important components of the innate immune system by detecting stressed and transformed cells and NK cell infiltration into tumors has been linked to better clinical outcome [83,100-102].

While a few studies highlight a link between p53 activity and NK cell regulation, no data is available for other p53 family members. Recently reactivation of wildtype p53 was described to improve lysis of tumor cells by NK cells [243]. Another study in mice highlighted p53 in the regulation of the activating NKG2D ligand, Rae-1ε, identifying p53 binding sites in the gene promoter region [244]. Furthermore, p53 activation in lung, breast and colon cancer cells led to an increase in NKG2D ligands on the cancer cells and more efficient NK cell-mediated killing of the tumor cells [245,246]. Interestingly, a study showed that in multiple myeloma cells activation of the DNA damage response pathway led to an increase in NKG2D ligands in a p53-independent manner. The ligand upregulation was however dependent on the functionality of the E2F-1 transcription factor [247]. Intriguingly, E2F-1 is known to activate p73 expression upon functional loss of p53 [214]. Based on this knowledge, we were interested to investigate if p73 isoforms are able to influence NKG2D ligand expression and thereby play a role in NK cell-mediated killing of tumor cells.

In cancer, a balance between tumor-suppressing and oncogenic p73 isoforms regulate the net outcome for the cell. In many solid cancers, the expression of ΔNp73, an oncogenic isoform of p73, is upregulated and overrules the effects of tumor-suppressing isoforms [201]. Therefore, we wanted to uncover the effects that the loss of ΔNp73 potentially has on NK cell ligand regulation.

As in Paper I, we utilized the TCGA breast cancer data set to evaluate possible correlations. Interestingly, we found a negative correlation between ΔNp73 expression and certain activating NK cell ligands, NKG2D ligands, MICB, ULBP1 and ULBP2. On the other hand, a positive correlation was detected for the NKG2D ligands MICA and ULBP3.

To validate the observed correlation, we performed a knock down of ΔNp73 in two human breast cancer cell lines (MCF-7 and MDA-MB-231) and studied the expression of NKG2D ligands. Knockdown of ΔNp73 in MCF7 cells resulted in increased mRNA and surface expression of MICA and MICB as well as ULBP2/5/6. Interestingly, in MDA-MB-231 cells mRNA levels of NKG2D ligands were significantly upregulated upon knockdown of ΔNp73. However, no detectable difference was observed in surface ligand expression. Cancer cells often find a way to avoid NK cell detection and consequent destruction. In the case of the MDA-MB-231 cell line, ligand shedding has been described as a common mechanism to evade

immune cell recognition [248]. This could explain the detected difference in mRNA versus surface expression on these cells.

To perform ΔNp73 knockdown, two siRNAs were used, recognizing different sequences of the mRNA of ΔNp73. These two siRNAs however resulted in similar ΔNp73 knockdown efficiency while showing differences in the effect on NKG2D ligand expression. Importantly, knockdown of ΔNp73 was detected on mRNA level using Taqman-based real time qPCR. In general, to detect the knockdown of a target, evaluation of the protein levels would be preferred, since posttranscriptional regulation might influence the total protein levels in the cell. Importantly, especially in the case of p73, posttranscriptional modifications and following degradation is the main regulatory mechanism of protein availability [249]. However, in the case of ΔNp73 endogenous protein detection is rather difficult. Actually, one of the biggest challenges of the p73 community remains the generation of a functional antibody against the ΔNp73 isoforms. Despite great efforts by us and others, so far, no antibody has been sufficiently specific.

Since our results indicate that ΔNp73 negatively regulates NKG2D ligand expression on breast cancer cells, we wanted to know what effects ΔNp73 knockdown has on NK cell-mediated killing of these cells. Unexpectedly, upon knockdown of ΔNp73 no difference was detected in the killing efficiency exerted by the NK cells. Importantly though, NK cells were able to kill both MCF7 and MDA-MB-231 cells, which can be concluded from the increasing proportion of dead tumor cells upon increasing effector-tumor ratios. However, the presence or absence of ΔNp73 in tumor cells did not change the amount of tumor cell killing.

To figure out what restrains NK cells from elevated killing efficiency even though NKG2D ligands are upregulated, we investigated if other NK cell ligands are also deregulated upon ΔNp73 knockdown. We were especially interested in the expression of inhibitory NK cell ligands. Once more, we used the TCGA breast cancer data set to compare patients with low and high ΔNp73 expression. And indeed, we found that low levels of ΔNp73 correlated with high expression of the inhibitory NK cell ligands HLA-A, HLA-B and HLA-C. This finding was further validated in various cell lines, where we found increased expression of HLA-A, HLA-B and HLA-C upon knockdown of ΔNp73. The concurrent upregulation of activating and inhibitory NK cell ligands could explain why no difference in NK cell-mediated tumor cell killing was detected. In a tumor setting, HLA expression is often downregulated to circumvent detection and killing by $CD8^+$ T cells, that use their T cell receptor to interact with HLA class I molecules presenting tumor antigens [250]. Therefore, an increase of HLA expression upon loss of ΔNp73 would suggest improved recognition of the cancer cells by $CD8^+$ T cells. *In vivo* experiments in immune competent mice need to be performed to investigate the net effects of simultaneous upregulation of activating and inhibitory NK cell ligands on tumor development.

In line with our findings, wild type p53 was described to be important in the upregulation of MHC class I molecules on the surface of tumor cells, which increased the visibility of the tumor cells to cytotoxic T cells [251]. Since ΔNp73 is able to inhibit p53 activity, ΔNp73 knockdown would release p53 from its inhibition and allow p53 to function, hence leading to an increase

in HLA expression on the tumor cells. However, as intriguing as this explanation sounds, our data points towards a p53-independent mechanism. When using a colon cancer cell line either wildtype or knockout for p53, upon knockdown of ΔNp73 HLA levels showed a trend to be upregulated in wildtype as well as in p53 knockout cells. Furthermore, ΔNp73-mediated upregulation of MICA and MICB was also found to be independent of p53 status. Interestingly though, overall MICA/B levels were lower in p53 deficient cells, indicating a role for p53 in NKG2D ligand regulation independent of ΔNp73.

Since the members of the p53 network can all influence each other, it would be interesting to see if other members or isoforms are involved in the regulation of NK cell ligands. Even though p53 family members share multiple transcriptional targets, they were all shown to have individual functions as well and bind to unique binding partners resulting in differential gene expression [156]. It is possible that p53 plays a role in NK cell ligand regulation independently of ΔNp73. However, it could be interesting to see if TAp73 has effects on NK cell ligand regulation in a competitive manner to ΔNp73. It would be possible that high levels of ΔNp73 inhibit TAp73-mediated expression of NK cell ligands and inhibition of ΔNp73 could release TAp73, allowing transcriptional activity.

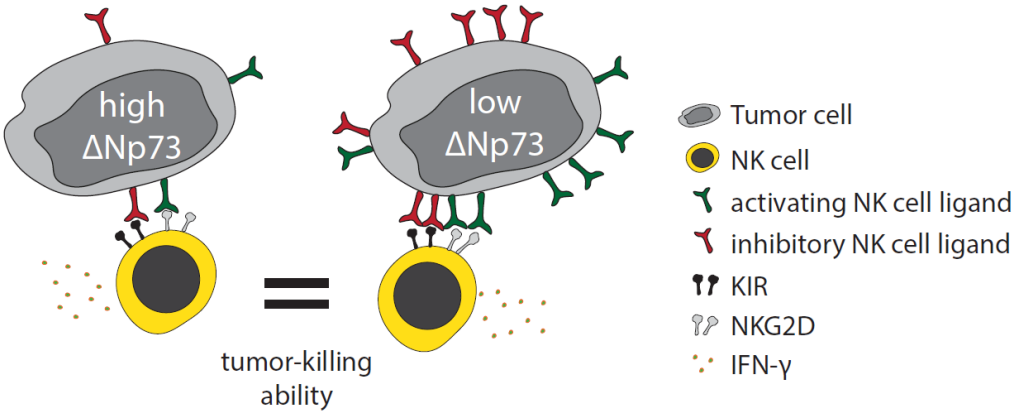

Figure 12. The effects of ΔNp73 levels in tumor cells on NK cell ligand expression and NK cell activity.

In conclusion, we discovered a role for ΔNp73 in the regulation of several NK cell ligands in breast cancer (Figure 10). Due to the activating and inhibitory nature of these NK cell ligands no difference in NK cell-mediated killing of tumor cells was detected. Furthermore, the exact mechanism how ΔNp73 negatively regulates NK cell ligands remains to be identified.

3.3 PAPER III

ΔNp73 enhances HIF-1α protein stability through repression of the ECV complex.

HIF-1α upregulation is a common feature of many human cancers, including breast cancer, and correlates with poor survival, see section 'Hypoxia' [252-257].

Various p53 family members have been described to influence the regulation of the HIF-1α pathway and angiogenesis. Several reports propose a complex interplay between the transcription factors p53 and HIF-1α on protein level. Interestingly, it was discovered that hypoxic conditions induce the stabilization of wildtype p53 in a HIF-1α-dependent manner. P53 was shown to associate directly with HIF-1α and to be transcriptionally active [258]. Later p53 was found to mediate proteasomal degradation of HIF-1α via recruiting the E3 ligase MDM2 [259]. Accordingly, p53 was found to suppress the transcription of HIF-1α target genes [260]. Interestingly, HIF-1α was found to compete with p53 for binding to the transcriptional co-activator p300/CBP thereby inhibiting its transcriptional activity [261]. Taken together, loss of p53 results in upregulation of HIF-1α target genes, due to a decrease in the competition for p300/CBP. On the other hand, severe hypoxia/anoxia induces increased p53 stability and thereby promotes HIF-1α degradation, most likely as a mechanism for cells to induce hypoxia-induced apoptosis.

Besides p53, ΔNp63, an oncogenic isoform belonging to the p63 proteins, has been identified as a regulator of angiogenesis in neuroblastoma and childhood osteosarcoma. ΔNp63 was found to induce HIF-1α stabilization and concomitant VEGF secretion via an IL-6/IL-8 – STAT3 pathway [262]. Conversely, the tumor suppressor TAp63 promotes the expression of Sharp-1, which associates with HIF-1α and induces its proteasomal degradation [263].

Likewise, also p73 proteins are known to regulate hypoxia and HIF-1α target gene expression. We have previously established a link between loss of TAp73 and increased angiogenesis and HIF-1α target expression [219]. At the same time another group reported similar findings. Amelio et al. confirmed that TAp73 opposes angiogenesis by interacting with the regulatory subunit of HIF-1α. This promotes the recruitment of MDM2 and leads to polyubiquitination and concomitant proteasomal degradation of HIF-1α [264]. These findings suggest that TAp73 can utilize the same mechanism as its family member p53 to promote HIF-1α degradation. Another study suggests that hypoxia-induced HIF-1α promotes the stability of the oncogenic p73 isoform, ΔNp73, by suppressing Siah1. Siah1 is an E3 ligase and was shown to be involved in the degradation of p73 proteins. Furthermore, ΔNp73 was shown to promote VEGF secretion and tumor angiogenesis [265]. At the same time the authors claim that also TAp73 stability is regulated by HIF-1α in the same manner. Namely, that under hypoxic conditions HIF-1α inhibits Siah1, which suppresses TAp73 degradation and leads to an increase of TAp73 levels and that this ultimately promotes tumor progression [266]. Even though these results are contrary to our own findings and those of others, it could be that the severity of oxygen deprivation in the tissue/cells plays a role how TAp73 is functioning, as it has been shown to be the case for

p53 [267]. Furthermore, spatial and temporal differences might be the reason for the opposing results.

While TAp73 deficient tumors displayed increased angiogenesis, we discovered that ΔNp73 loss was accompanied by a drastic decrease in angiogenesis and reduced tumor growth [219]. Additionally, another group also reported reduced vessel formation and tumor growth in ΔNp73 deficient tumors compared to wildtype tumors [265]. Contrary to the disputed role of TAp73 in angiogenesis regulation, a promoting role for ΔNp73 in the angiogenic process is well established. This being said, so far no one has investigated if ΔNp73 regulates angiogenesis by opposing p53/TAp73s regulation of HIF-1α or if it does so in a different manner. We were therefore intrigued to uncover the underlying regulatory mechanism how ΔNp73 regulates HIF-1α.

To do so we knocked down ΔNp73 stably using shRNA or transiently with siRNA, in breast cancer cell lines MCF-7 and MDA-MB-231. ΔNp73 knockdown led to a reduction of HIF-1α protein levels and consequently reduced HIF-1α target gene expression. Interestingly, decreased HIF-1α protein levels were observed under hypoxic and normoxic conditions, indicating an oxygen-independent regulation by ΔNp73.

To study the effect of ΔNp73 on HIF-1α regulation *in vivo* we injected nude mice with transformed MEFs, either wildtype or deficient for ΔNp73. HIF-1α protein levels were found to be significantly lower in ΔNp73 knockout tumors even after normalization to tumor size. HIF-1α levels were investigated in hypoxic and non-hypoxic tumor areas. We found lower HIF-1α levels in ΔNp73 knockout tumors in both hypoxic and non-hypoxic areas, compared to wildtype tumors.

Importantly, neither ΔNp73 knockdown nor overexpression affected HIF-1α mRNA expression. Inhibiting the 26S-dependent proteasomal degradation machinery we discovered that ΔNp73 interferes with the protein stability of HIF-1α. Furthermore, we found increased levels of ubiquitin bound to HIF-1α in ΔNp73 knockout cells and upon knockdown of ΔNp73 in human breast cancer cells.

To figure out how ΔNp73 regulates proteasomal degradation of HIF-1α we compared HIF-1α hydroxylation but could not detect any difference between wildtype or ΔNp73 deficient cells. Next, we utilized the human renal cell line RCC4, which normally is VHL deficient but is also available as a version that has VHL reintroduced ($RCC4^{+VHL}$). Knockdown of ΔNp73 in RCC4 cells did not show any effect on HIF-1α protein levels. However, in $RCC4^{+VHL}$ cells knockdown of ΔNp73 led to significantly decreased protein levels of HIF-1α, indicating that ΔNp73 interferes with VHL-mediated regulation of HIF-1α. However, no differences in VHL mRNA or protein levels were detected upon knockdown of ΔNp73. While VHL seems to be crucial for HIF-1α regulation by ΔNp73, the results point out that ΔNp73 does not influence HIF-1α via regulating VHL levels.

To evaluate which pathways are deregulated by ΔNp73, we used the TCGA breast cancer data set and compared samples with low and high ΔNp73 expression. Interestingly, we found ubiquitin-mediated proteolysis upregulated in samples expressing low levels of ΔNp73. Furthermore, we confirmed our previous findings that ΔNp73 does not affect VHL or HIF-1α mRNA expression levels.

Several of the genes downregulated upon high levels of ΔNp73 were genes involved in the ECV complex. We validated the expression of mRNA levels of Elongin B (TCEB2), Elongin C (TCEB1), Cullin 2 (CUL2) and Rbx1 (RBX1) and found that all of them were upregulated upon ΔNp73 knockdown. Conversely, upon ΔNp73 overexpression the members of the ECV complex showed decreased mRNA expression levels. Furthermore, we confirmed elevated protein levels of these ECV genes in ΔNp73 deficient cells compared to wildtype. Analysis of the promoter regions of the ECV genes identified putative p53/p73 binding sites, which ΔNp73 would be able to compete for binding to, thereby inhibiting transcription. By performing chromatin immunoprecipitation, we were able to validate that ΔNp73 binds to the promoter region of RBX1 and TCEB1. Rbx1 is the RING finger protein that together with Cullin 2 is necessary for the polyubiquitination of HIF-1α. By silencing the E3 ligase Rbx1, we confirm that Rbx1 is essential for HIF-1α degradation. Finally, knocking down both Rbx1 and ΔNp73 simultaneously did not lead to a decrease in HIF-1α levels, suggesting that the enhanced degradation of HIF-1α in absence of ΔNp73 is due to enhanced Rbx1 activity.

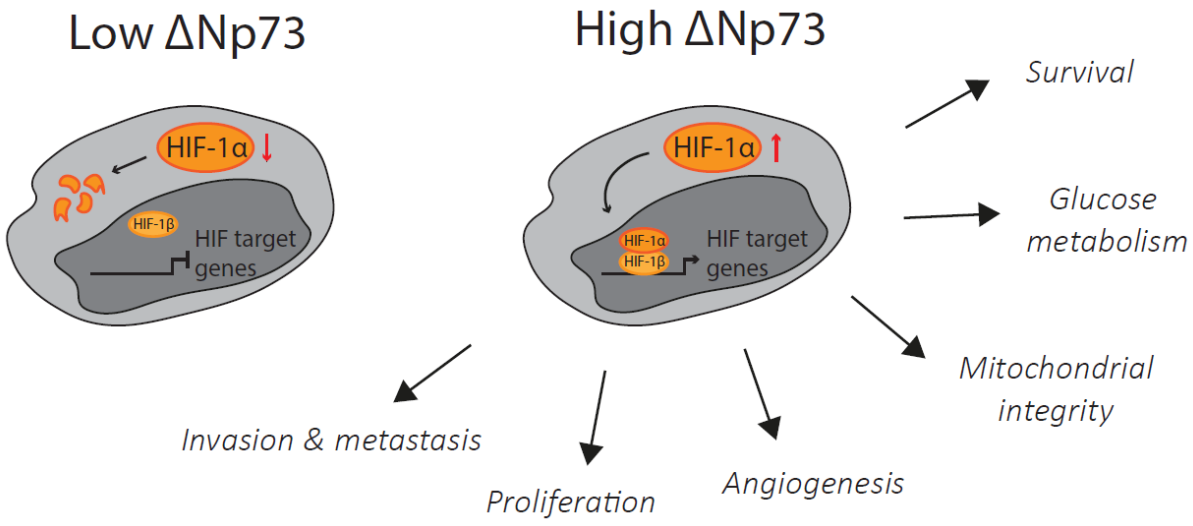

Figure 13. ΔNp73 induces HIF-1α stability and expression of HIF-1α target genes.

In conclusion, we identify ΔNp73 to be an important regulator of the HIF-1α transcription factor. ΔNp73 inhibits expression of members of the ECV complex and thereby promotes HIF-1α protein stability. ΔNp73 therefore acts through a mechanism unrelated to the one its family member TAp73 utilizes to regulate HIF-1α. Overall, these findings once again strengthen ΔNp73s role as an oncogenic protein able to influence multiple cellular processes involved in tumor progression.

3.4 PAPER IV

ΔNp73 regulates the expression of the multidrug-resistance genes ABCB1 and ABCB5 in breast cancer and melanoma cells - a short report.

Our previous findings that high ΔNp73 expression in breast cancer patient biopsies correlates with angiogenesis and hypoxia left us curious to investigate which other pathways are differentially regulated in high versus low ΔNp73 expressing samples. Therefore, we utilized the TCGA BRCA data set and performed a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on the genes that were at least 2-fold up- or downregulated in ΔNp73 high samples compared to samples without detectable ΔNp73 expression. Interestingly, we identified the ABC transporter pathway as the most upregulated pathway in ΔNp73 high expressing samples.

ABC transporters are surface glycoproteins that catalyze the export of lipids, metabolic products and other small molecules, such as drugs, through intra- and extracellular membranes [268,269]. Cancer cells commonly upregulate ABC transporters as a mechanism to resist killing by chemotherapeutic drugs and confer multidrug resistance (see section 'Multidrug Resistance') [154]. Importantly, ABC transporter expression correlates with poor prognosis and drug failure in many types of cancer [270].

Our analysis showed that the most significantly upregulated genes in the ΔNp73 high expressing samples belonged to the ABC families A (ABCA), B (ABCB), C (ABCC) and G (ABCG), with ABCB5 being the highest upregulated gene.

To validate our findings that ΔNp73 correlates with ABC transporter expression we overexpressed ΔNp73α in the breast cancer cell lines MCF-7 and MDA-MB-231. While we could not detect any expression of ABCA8, ABCA9 and ABCA10, we were able to detect an increase in ABCB1 and ABCB5 mRNA levels. Conversely, by performing transient or stable knockdown of ΔNp73 we confirmed a decrease in ABCB1 and ABCB5 expression compared to controls.

ABCB1, commonly referred to as multidrug resistance gene 1 (MDR1) or g-glycoprotein (g-gp), has been shown to correlate with poor response to chemotherapy in breast cancer [271]. In melanoma, chemoresistant cells are defined by their increased expression of ABCB1 and ABCB5 [272]. Treatment with chemotherapeutic drugs led to the selection of cells upregulating ABCB5 and this data was supported by the analysis of clinical samples of melanoma patients that received the chemotherapeutic drug dacarbazine [273].

The participation of p53 family members in the regulation of ABC transporter is well established. Previously, p53 has been shown to play a role in the regulation of ABCB1 expression. Loss of functional p53 resulted in elevated levels of ABCB1 [274].

Furthermore, ΔNp73α has been described to cause upregulation of ABCB1, by interfering with p53-mediated transcription in gastric cancer [275]. So far however, our data for the first time present a clear correlation between ΔNp73 and ABC transporters in breast cancer.

Next, we were interested to investigate if the ΔNp73-mediated upregulation of ABC transporters effects cellular responses to drug treatments. Therefore, we treated MCF-7 or MDA-MB-231 cells, either control or stable knockdown for ΔNp73, for 30 minutes with Doxorubicin and evaluated drug retention directly after or 3 hours after drug treatment. Intriguingly, we discovered that cells containing a stable knockdown for ΔNp73 retained more of the drug in the cytosol, indicating lower efflux ability. Additionally, cells containing a stable knockdown for ΔNp73 showed decreased cell proliferation upon treatment with Doxorubicin in a dose-dependent manner. These results suggest that ΔNp73 is actively involved in regulating resistance to chemotherapeutic drugs in breast cancer by inducing increased expression of ABC transporters (see Figure 14).

Considering the strong link between ABCB5 and self-renewal, differentiation and drug resistance in melanoma [273,276], we were intrigued to investigate if ΔNp73 regulates ABC transporter expression also in melanoma. Importantly, the predominantly expressed oncogenic p73 isoform in melanoma is p73ΔEx2/3 [216]. In line with this, in biopsies from melanoma patients we could detect the expression of p73ΔEx2/3, however no expression of ΔNp73 was detectable. We then correlated p73ΔEx2/3 expression with the expression of ABCB1 and ABCB5 in these melanoma samples. ΔNp73 correlated with the expression of both genes, however, only the correlation with ABCB5 was statistically significant.

To confirm these findings, we overexpressed p73ΔEx2/3α or p73ΔEx2/3β in the SK-MEL-28 human melanoma cell line. As expected, ABCB1 and ABCB5 expression was increased upon overexpression of different p73dEx2/3 isoforms. Thus, we confirmed a link between ΔNp73 and multidrug resistance genes also in melanoma.

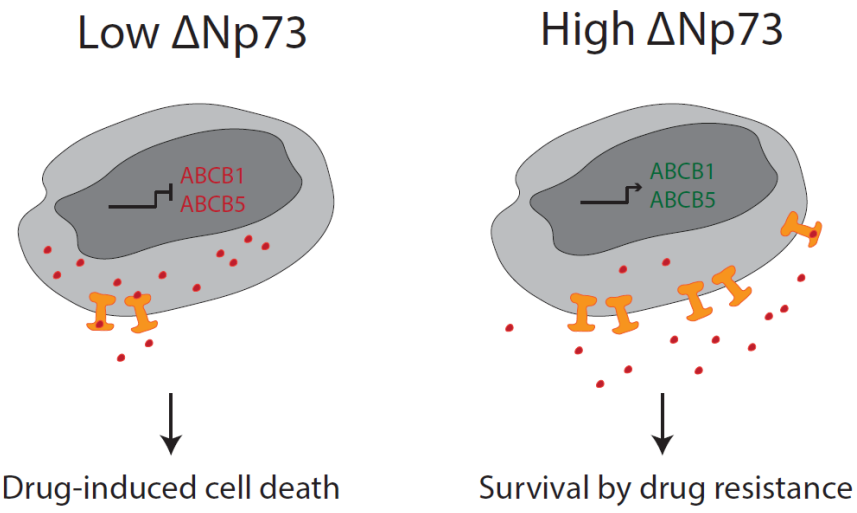

Figure 14. ΔNp73 promotes multidrug resistance through inducing ABC transporters

Considering that p53 proteins have previously been shown to regulate multidrug resistance genes and that ΔNp73 has been described to inhibit p53-dependent repression of ABC transporters in other types of cancer, the underlying mechanism will most likely be the same in breast cancer. It would however be interesting to see if TAp73 holds a similar regulatory function as p53, competing with ΔNp73.

4 CONCLUSION AND FUTURE PERSPECTIVES

In the current thesis, we identified several novel ways by which p73 proteins control the tumor microenvironment (see Figure 15). In Paper I, we established an inhibitory role for TAp73 on the NF-κB pathway and we presented that the loss of TAp73 led to an upregulation of NF-κB activity and a simultaneous increase in macrophage infiltration into the TME. In Paper II, the oncogenic p73 isoform, ΔNp73, was found to influence the expression of various NK cell ligands. However, due to the concomitant upregulation of NK cell ligands of activating and inhibitory nature, no overall effect on NK cell activity and NK cell-mediated killing of tumor cells was detected. In Paper III, ΔNp73 was shown to induce HIF-1α protein stability. We revealed that ΔNp73 inhibits the transcription of genes comprising the ECV complex, which otherwise polyubiquitinates HIF-1α, leading to its proteasomal degradation. Finally, in Paper IV, a correlation between high levels of ΔNp73 and the upregulation of ABCB1 and ABCB5 was established in breast cancer and melanoma. These two genes belong to the ABC transporter family which is known to be involved in multidrug resistance of cancer cells.

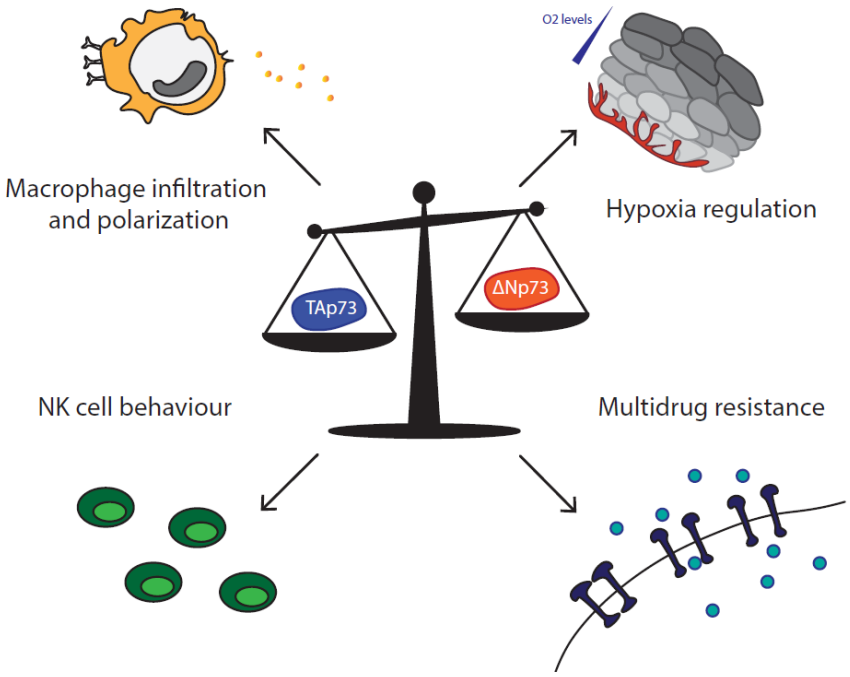

Figure 15. Novel implications of p73 proteins in regulating the TME

The different members of the p73 family are known to regulate a multitude of processes in cancer development and progression. While full-length p73 isoforms, TAp73, were shown to play a role in apoptosis, genetic stability, migration and angiogenesis, the N-terminally truncated isoforms, ΔNp73, possess oncogenic characteristics promoting cancer stemness, drug resistance and EMT, hence fostering invasion and metastasis [218,219,264,277-280]. Most studies so far have focused on what effects the expression or loss of different p73 isoforms have on the cancer cell itself. However, how the expression of different p73 isoforms in cancer cells

influences the immune cell compartment in the TME has so far not been studied. In Paper I and Paper II, we identified at least two different immune cell types that are affected by p73 isoforms. In Paper I, we found that loss of TAp73 increased the recruitment of macrophages to the TME and these macrophages showed a tumor-promoting activation state. In this study we also describe how TAp73 inhibits NF-κB activity. Since the NF-κB pathway is a major transcriptional regulator of inflammatory processes it is quite likely that TAp73 loss also affects other immune cell types. In our analysis of the TCGA breast cancer data set, we found that most genes upregulated in samples expressing low levels of TAp73 belonged to pathways related to immune regulations. Furthermore, several of these genes belong to the group of cytokines and chemokines, which are responsible for the recruitment of immune cells. Based on these facts, TAp73 loss seems to, at least indirectly, influence more than just macrophage infiltration. However, this remains to be investigated experimentally.

Additionally, in Paper II, we discovered that loss of ΔNp73 induces the upregulation of several NK cell ligands on cancer cells. Various activating NKG2D ligands as well as inhibitory KIR ligands were found to be increased simultaneously upon loss of ΔNp73. Even though we did not observe any difference in NK cell-mediated tumor cell killing in an *in vitro* co-culture set-up, it cannot be excluded that the scenario *in vivo* would result in a different outcome. This hypothesis stems from the fact that NKG2D, an activating receptor, is not only expressed on NK cells but also on various T cell populations. It might therefore be that if we target ΔNp73 *in vivo* the accompanied increase of NKG2D ligands might stimulate the activity of T cell populations resulting in enhanced tumor killing. Furthermore, the increase of HLA molecules upon loss of ΔNp73 could also activate $CD8^+$ T cells, inducing tumor killing. However, without proper *in vivo* experiments these ideas remain purely speculative. Nonetheless, based on the results of Paper I and Paper II, it can be concluded that p73 proteins take up an important role in regulating parts of the immune cell compartment in cancer, thereby influencing the TME.

In Paper III, we describe a novel mechanism how ΔNp73 regulates HIF-1α protein stability. ΔNp73 was found to suppress the expression of various genes of the regulatory machinery involved in HIF-1α degradation. Several members of the p53 family have been previously reported to contribute to the regulation of HIF-1α. Importantly, the identified mechanism of ΔNp73 is distinct from the one TAp73 and p53 have been described to use to control HIF-1α. Our findings highlight the complexity within the p53/p73 family. In the case of HIF-1α, it can be concluded that all members of the p53 family contribute to its regulation, indicating a certain redundancy, as well as the existence of regulatory mechanisms between the members.

Paper IV illustrates the correlation between ΔNp73 and the multidrug resistance genes, ABCB1 and ABCB5, in breast cancer and melanoma. Interestingly, a recent study reported a HIF-1α-mediated increase of ABCB1 and ABCB5 in ovarian cancer. HIF-1α was shown to bind HRE in the ABCB1 and ABCB5 promoters and activate their transcription [281]. These results link Paper III with Paper IV. High levels of ΔNp73 correlate with increased HIF-1α protein stability as well as increased ABCB1 and ABCB5 expression. Therefore, it is possible that the underlying mechanism how ΔNp73 initiates ABC transporter expression is that ΔNp73 induces

HIF-1α stability, which in turn activates ABC transcription. This is an attractive theory since inhibition of HIF-1α was shown to reverse multidrug resistance in colon cancer cells through a decrease of ABCB1 [282]. Furthermore, another study suggests an increased activity of the NF-κB pathway in a subset of melanoma cells, which expressed high levels of ABCB1 and ABCB5 [272]. Strikingly, the NF-κB pathway has been described to induce ABCB1 expression via direct binding to a NF-κB response element in the first intron of the ABCB1 gene [283]. Considering these results, it might be that both loss of TAp73, via an increase in NF-κB activity, and increased levels of ΔNp73, via elevated HIF-1α stability, can lead to the upregulation of ABC transporters and thereby influence multidrug resistance.

Our studies provide strong evidence for p73 proteins being important in controlling the TME. The fact that p73 proteins can hold opposing functions in cancer and regulate each other's expression levels makes investigating the role of individual isoforms a rather challenging task. A complex balance of all p73 isoforms, as well as other members of the p53 family, defines cellular outcome. Interestingly, in Paper I, we propose that TAp73 is competing with NF-κB for the transcriptional co-activator p300/CBP, thereby inhibiting the transcription of NF-κB target genes. All members of the p53 family have been shown to rely on binding to this co-factor for transcriptional activity [226,227,230,261]. Furthermore, p300/CBP has also been shown to interact with HIF-1α and as mentioned previously, with NF-κB [284]. This competition between different transcription factors for transcriptional co-activators adds another layer of complexity to an already complex network of interactions and regulation within the p53 family.

P73 proteins are involved in a multitude of different processes regulating cancer development and progression, covering most of the cancer hallmarks defined by Hanahan and Weinberg (see Figure 1). Targeting distinct p73 isoforms, and thereby shifting the balance, would affect several processes simultaneously, instead of focusing on a single hallmark. Furthermore, since p73 isoforms are important during normal development but are expressed at rather low levels in healthy tissues in adults, targeting these isoforms might be well tolerated. In cancer, overall p73 levels are increased compared to healthy tissue. Promoting TAp73 activity or decreasing ΔNp73 levels in cancer cells could therefore have great therapeutic effects. However, so far, no promising strategies have been developed that could be used in the clinics.

The significant involvement of the p53 family network in cancer makes it indispensable to understand how the different members interplay. Especially since there is a great deal of redundancy between p53 family members, which needs to be understood to be able to therapeutically target the network and achieve lasting results.

In conclusion, the papers comprising this thesis aid in understanding the functions of different p73 isoforms in regulating the tumor microenvironment and lay ground for further investigation in the field.

5 ACKNOWLEDGEMENTS

First of all, I want to thank my main supervisor **Margareta Wilhelm**. **Maggan**, over the years you have been a true inspiration. Your knowledge and dedication when it comes to research is unbelievable. Besides to discuss experiments I knew your door was always open and you would listen to any problems I had. Even though we didn't agree on everything, especially color panels for figures, we still managed to get everything done. I wish you all the success that you deserve with your research and that you keep such a nice group going. Thank you for all your support!

To my co-supervisors, **Charlotte Rolny**, **Andreas Lundqvist** and **Dhifaf Sirhan**: With some of you I had more contact, some less. I really valued all your input on the different projects and providing me with information and tools on immunology. **Charlotte** for collaborating and helping out a lot with the macrophage project. **Andreas** for always providing us with antibodies when we needed them last minute. And **Dhifaf**, especially to you, thank you so much for all your time and support. You always managed to find time to discuss my projects with me. Good luck for all your future steps!

To all current lab members: **Vero**, from the first day you joined the lab I felt this positive vibe that you bring with you everywhere you go. I really enjoyed our conversations in English, Spanish or Swedish, depending on how we felt that day. Also, I already miss going to Absolution with you on Thursdays. I always enjoyed taking this break with you! **Niek**, I am so happy you decided to come back to do your PhD in our lab. I really enjoyed our lunches together looking at pictures of the newest additions to your parents' farm. Thank you for always helping out with absolutely everything! Good luck with your PhD! **Leilei**, I really appreciated your friendly smile every time we met in the lab and the cute emojis you added in your emails! I wish you good luck for your future! :)

To all previous members of the lab: A big thank you, **Habib**, for helping me getting started with the project. Teaching me a lot in the lab and introducing me to possibilities such as the MSA and KIcancer. I really missed your calm spirit in the lab after you left! **Marina**!! Thanks for everything! You really helped me a lot throughout the years. I was always afraid that you might leave one day and then you did. We miss you in the lab! Wish you all the best for your future! **Ana**, I'm so glad to see you so happy again! It gave me motivation and showed me that there is life after PhD. Also, you are the reason I started playing Squash. I really want to thank you for this because it has been almost therapeutic to go and smash a ball against a wall after an exhausting day in the lab. **Robin**, I include you here, because it really feels like you've been part of our lab. I mean at some point you just showed up in our office sitting next to me every day. Good luck for your future!

I want to thank my mentor at KI, **Julian Walfridsson**. Thank you for your support and all the discussions about future possibilities. I really valued our meetings, to me it almost felt like two friends talking about life.

Not to forget, my almost co-supervisor **Mikael Karlson**. **Micke**, thank you for taking me into your group meetings and journal clubs (even though I have been lousy in attending them during the last few months). I really appreciated your input and that you were always eager to help in any way you could. Thank you for that! Also a big thanks to all the group members, for nice discussions and suggestions for my projects!

Kiran and **Nicolas**, thanks for all your input and the help you provided me with my projects. It's been great working with both of you! Also, a big thank you to **Jonathan** and **JJ** for their collaborations! :)

Thanks to all collaborators and co-authors! Without you it wouldn't have been possible!

I want to thank all the students that helped me out during my time in the lab. I hope you learned as much as I did during your stay. Thank you, **Janina**, **Larsen**, **Aitor** and **Mark**!

A big thank you to **Marie Arsenian Henriksson** and all members of her group! For all the valuable discussions during our joint meetings. **Mariavi, Aida, G, Mustaq, Vittoria,** thank you for all the fika we had together. **Lourdes**, a special thanks to you! It has been great being your friend. I really appreciate that you were there for me when I needed a friend! <3

To my MTC friends that finished their PhD before me and left to find happiness in new places: It hasn't been the same here without you guys! **Silke**, thank you for making me join SATS and introducing me to Cardio ENERGY. Thanks for all the ESA sushi lunches and wine or movie nights. Thanks for all the fika and the motivation you gave me to finish. You're so caring and thoughtful! I can't thank you enough to take me into your home when I needed it the most. <3 **Mitch**, it's been great to have a fellow Austrian around. Thank you for bringing Silke back to Vienna! I'll see you there! **Vanessiña**, my friend! You've taught me so much. I love discussing the world with you. Thank you for taking me with you to Stockholm Food Movement and other insightful evening events, widening my horizon. I can't wait to meet you in Germany or have another trip together to Portugal! <3 And last but not least, **Nestito**! Best roomie. I'm extremely grateful for our friendship, for our endless discussions about life and love, and not to forget, for all balcony evenings we had together. I miss you, amigo!

To all the nice people I met at MTC: **Wesam**! You were always there for me when I needed help or someone to talk to, and also anytime I felt like eating ice-cream. I really appreciate that! Hope you come and visit me in Vienna. **Benedek**, sometimes western medicine needs a touch of traditional treatments. Go try acupuncture, I'm quite sure you'd enjoy it. I love it! Thanks for all the awesome parties at your place and our hiking and biking trips. I only have the best memories! **Sharesta**, thank you for being such an open and joyful person. Thanks for all our fika and climbing sessions. I wish you all the happiness for your future! <3 **Mariana**, thank you for fika, ESA sushi lunches, dinner at your place and the trip to 'your' summer house! Too bad our plan of travelling South America together didn't work out. Maybe at some point we manage to do it after all. Thank you, **Leona**, **Julian** and **Chris**, I always enjoyed meeting you guys in the animal house! **Shady**, thank you for all the conversations we had on how we

imagine life, work and our future! With your calm spirit you've always helped me look at things from a different angle, from where they only looked half as bad. **Leonie**, I want to thank you for being the person you are. So caring and helpful! Good luck with the rest of your PhD. **Basile**, thank you for being there for me when I needed you!!! I won't forget that! **Nicol(a)ina**, it's been great spending almost a year with you being around. I miss our weekly pancakes and daily ping pong sessions. **Twana**, I have to tell you I'm always happy to see you. You are such a calm, glad person, which just makes it great being around you! Keep up this spirit. **Lidia**, **Gergana**, **Tanzina**, **Okan**, **Pavitra**, thank you for always helping me out. You've all been great company in the lab. **Graciela**, thanks for our short collaboration! I really enjoyed the time :). **Katrine,** thank you for always helping out with antibodies!! **Ainhoa**, I always enjoyed our spontaneous chats at the coffee machine! Good luck with your PhD. **Marco**, thanks for taking over the MSA. Someone had to do it! ;) **Sanjana**, **Pradeepa**, **Ruining**, thanks for engaging in the MSA! I had a great time with you guys there! **Patrick**, we've only had a short time together in the beginning, but it was a good time and I like to remember it! Thank you :) **Carina**, thanks for always being so welcoming! I wish you and your family all the best. **Carol**, I like to remember the good times we had inside and outside the lab. And I'm really glad we managed to stay in contact afterwards! :)

I also want to thank **Gesan**, **Eva** and **Åsa**, for always helping me out and making my PhD life easier. I couldn't have wished for better support!

To my friends at KI: Thank you, **Mirco**, for being such an inspirational person. You are so driven by doing good and creating a better place for everyone here at KI. It's been a joy to be part of this and see you in action. Thanks for always caring! **Henna**, it's been great having you live in the same house. For joint Sunday breakfasts, dinners or movie nights! Gracias, **Angeles** cariña, for all the listening and for your trust in me. I am so thankful for your support and I wish you all the best in the world. You deserve it so much! <3 **Huthayfa**, thanks for being my friend for so many years! Thanks for all the parties and also thanks for always helping out when I needed it. **Aurelie** it has been great having you around. I'll never forget when I met you at this random party in Vienna, the world is so small. You're such a cheerful person and I wish you all the best, whatever your path will be! **Sissi**, thank you for nice brunches/lunches and funny talks. You've always managed to cheer me up! **Felix**, we basically just met, but I can already say that I will miss you! **Vincent** and **Irene**, thank you for being my Austrian and Spanish speaking friends. I'm so happy you guys moved to Vienna! It showed me that this step is possible :D See you there! **Eliane**, we met at the very beginning, more than 7 years ago. So much has changed over all these years, but I'm so happy to still call you my friend! **Susi**, thank you for being who you are. Your cheerful character mixed with the cocky jokes always made me laugh. Good luck for your future steps! Thank you, **Tatjana**, for our collaborations in the beginning and the friendship in the end. I feel like I have learnt a lot from you! **Sunjay**, thanks for many parties and our trip to Greece. I have great memories from it! Thank you, **Michalis**, for being part of my journey here in Sweden from the very start. I hope our paths keep on crossing! Good luck with everything!

Fadwina, I don't know how to put this down in words. We've been through a lot together and even though we sometimes manage to not see each other for months, I know you are always there for me. I'm deeply grateful for your friendship. Love you, my little Fadwina!

To my friends from the master: **Jaime**, it's been great having you around and discussing advantages and disadvantages of living in Sweden, as well as sharing books. I hope we keep up meeting in Stockholm and Vienna! Good luck finishing your PhD, you're almost there! **Uta**, I'm happy we spent so much time together lately! I really enjoyed our long walks and talks. I wish you all the strength to finish and move to the next step, wherever it will be. Thank you, **Johanna**, for being the first person really speaking Swedish to me. Thanks for taking us to a proper Swedish midsummer and all the nice memories from our years together. **Mona**, finally it seems we both made it. It's been hard times, but we got through it! Thanks for always keeping in contact. I hope we manage to meet more often in the future! :) **Oscar**, thanks for our lunches. I wish you all the best with the rest of your PhD! **Eleni and Vasilis**, I am so glad to have met you. You are two of the happiest people I know. Always positive and so helpful! I will never forget your great hospitality when I joined your wedding in Greece. Stay as you are, my friends <3

And finally, … what would I have done without you, **Nati**?! We basically know each other since the first day I arrived in Sweden and have been friends ever since. Moving closer and closer to each other, in the end we managed to move in together and it has been the best time of all these years! Your happy/slightly-to-very-crazy spirit always makes me feel better. Our evening dancing sessions can't be replaced by anything better. And our travel to Norway was most likely the most amazing trip one can ever imagine. Even though I will leave, I want you to know that there will always be an extra room for you in my place! I love you, Natimaus! <3

To my friends back home: **Tamtam, Gugi, Ingo, Tom, Ines, Patci, Heli, Nora, Georg**. You can't imagine how lucky I consider myself to have all of you in my life! Even though I've been horrible in keeping contact during most of the time I spent in Sweden, every time we met in person all of you just made me feel like I've never left. Can't wait to meet you all more often soon! <3

To my **family**: No words can express how grateful I am! **Mom**, **Dad**, I wouldn't be here without you. Thanks for believing in me and making me feel that I can achieve anything! I love you! **Jutschi**, I couldn't wish for a better sister. You're my best friend! Thank you for always being there for me. And of course, a massive thank you to **all family members** and extended family members that have been supporting me throughout all my life! I'm so happy to call you my family! <3

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